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Method for the production of polyunsaturated fatty acids in plants

Description

The present invention relates to a method for the production of fatty acid esters which comprise unsaturated fatty acids with at least three double bonds, and to free unsaturated fatty acids with a content of at least 1% by weight based on the total fatty acids present in the plants, by expressing at least one nucleic acid sequence which encodes a polypeptide with Δ6-desaturase activity and at least one nucleic acid sequence which encodes a polypeptide with Δ6-elongase activity. Advantageously, these nucleic acid sequences can, if appropriate, be expressed in the transgenic plant together with a third nucleic acid sequence which encodes a polypeptide with Δ5-desaturase activity.

The invention furthermore relates to the use of defined nucleic acid sequences which encode polypeptides with a Δ6-desaturase activity, Δ6-elongase activity or Δ5-desaturase activity selected from a group of nucleic acid sequences, and/or to the use of nucleic acid constructs comprising the abovementioned nucleic acid sequences.

Certain products and by-products of naturally occurring metabolic processes in microbial cells or in the cells of animals and, advantageously plants, have utility for a wide range of industries, including the feed, food, cosmetics and pharmaceutical industries. These molecules, which are collectively termed "fine chemicals", also include, for example, lipids and fatty acids, one representative class of which are the polyunsaturated fatty acids. Polyunsaturated fatty acids (PUFAs) are added for example to infant formula for increasing the nutritional value of these foods. PUFAs have, for example, a positive effect on the cholesterol level in the blood of humans and are therefore useful for protection against heart disease. Fine chemicals such as polyunsaturated fatty acids (PUFAs) can be isolated from animal sources such as, for example, fish, or produced by microorganisms by culturing microorganisms which have been developed such that they produce and accumulate or secrete large amounts of one or more desired molecules.

Fatty acids and triglycerides have a multiplicity of uses in the food industry, in animal nutrition, in cosmetics and in the pharmacological sector. Depending on whether they take the form of free saturated or unsaturated fatty acids or triglycerides

with an increased content of saturated or unsaturated fatty acids, they are suitable for a variety of uses. Polyunsaturated Ω 3-fatty acids and Ω 6-fatty acids constitute an important part of animal and human nutrition. Owing to the present-day composition of human nutrition, an addition of polyunsaturated Ω 3-fatty acids, which are predominantly found in fish oils, to the food is of particular importance. Thus, for example, polyunsaturated fatty acids such as docosahexaenoic acid (=DHA, $C22:6^{\Delta 4,7,10,13,16,19}$) or eisosapentaenoic acid (= EPA, $C20:5^{\Delta 5,8,11,14,17}$) is added to baby formula for increasing the nutritional value. DHA is said to have a positive effect on brain development.

The various acids and triglycerides are obtained mainly from microorganisms such as Mortierella or from oil-producing plants such as soybeans, oilseed rape, sunflower, algae such as Cryptocodinium or Phaeodactylum and others, the products being obtained, as a rule, in the form of their triacylglycerides (= triglycerides = triglycerols). However, they can also be obtained from animals such as, for example, fish. The free fatty acids are advantageously prepared by hydrolysis. Higher polyunsaturated fatty acids such as DHA, EPA, arachidonic acid (= ARA, $C20:4^{\Delta 5,8,11,14}$), dihomo-γ-linolenic acid ($C20:3^{\Delta 8,11,14}$) or docosapentaenoic acid (DPA, $C22:5^{\Delta 7,10,13,16,19}$) cannot be isolated from oil crops such as oilseed rape, soybeans, sunflower, safflower or others. Conventional natural sources of these fatty acids are fish such as herring, salmon, sardine, redfish, eel, carp, trout, halibut, mackerel, zander or tuna, or algae.

Depending on the intended purpose, oils with saturated or with unsaturated fatty acids are preferred; thus, for example, lipids with unsaturated fatty acids, specifically polyunsaturated fatty acids, are preferred in human nutrition. The polyunsaturated Ω 3-fatty acids are said to have a positive effect on the cholesterol level in the blood and thus on the possibility of preventing heart disease. The risk of heart disease, stroke or hypertension can be reduced markedly by adding these ω3-fatty acids to the food. Also, Ω 3-fatty acids can have a positive effect on inflammatory processes, specifically chronically inflammatory processes in connection with immunological diseases such as rheumatoid arthritis. These fatty acids are therefore added to foodstuffs, specifically dietetic foodstuffs, or are used in medicaments.

In connection with these rheumatic diseases due to the usual composition of our foods, Ω 6-fatty acids such as arachidonic acid tend to have a negative effect on these diseases.

Ω 3- and Ω 6-fatty acids are precursors of tissue hormones, what are known as eicosanoids such as the postaglandins, which are derived from dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid, the thromoxanes and the leukotrienes, which are derived from arachidonic acid and eicosapentaenoic acid. Eicosanoids (known as the PG_2 series), which are formed from Ω 6-fatty acids, promote, as a rule, inflammatory reactions, while eicosanoids (known as the PG_3 series) from Ω 3-fatty acids have a minor, or no, proinflammatory action.

Owing to the positive properties, there has been no lack of attempts in the past to make available genes which are involved in the synthesis of fatty acids or triglycerides, for the production, in various organisms, of oils with a modified content of unsaturated fatty acids. Thus, WO 91/13972 and its US equivalent describe a Δ9—desaturase. A Δ15—desaturase is claimed in WO 93/11245 and a Δ12—desaturase is claimed in WO 94/11516. Further desaturases are described, for example, in EP—A—0 550 162, WO 94/18337, WO 97/30582, WO 97/21340, WO 95/18222, EP—A—0 794 250, Stukey et al., J. Biol. Chem., 265, 1990: 20144—20149, Wada et al., Nature 347, 1990: 200—203 or Huang et al., Lipids 34, 1999: 649—659. However, the biochemical characterization of the various desaturases is incomplete as yet since the enzymes, being membrane-bound proteins, can only be isolated and characterized with great difficulty (McKeon et al., Methods in Enzymol. 71, 1981: 12141—12147, Wang et al., Plant Physiol. Biochem., 26, 1988: 777—792). As a rule, membrane-bound desaturases are characterized by introduction into a suitable organism which is subsequently analyzed for enzyme activity by means of analyses of the starting material and the product. Δ6—Desaturases are described in WO 93/06712, US 5,614,393, US5614393, WO 96/21022, WO00/21557 and WO 99/27111, and their application for the production in transgenic organisms has also been described, such as in WO98/46763 WO98/46764, WO9846765. In this context, the expression of various desaturases is also described and claimed, as is the case in WO99/64616 or WO98/46776, as is the formation of polyunsaturated fatty acids. As regards the efficacy of the expression of desaturases and their effect on the formation of polyunsaturated fatty acids, it must be noted that only minor contents of Δ6-unsaturated fatty acids/lipids, such as, for example, gamma-linolenic acid and stearidonic acid, have been obtained by expression of a single desaturase, as described to date. Moreover, a mixture of ω 3- and ω 6-fatty acids has been obtained as a rule, since all of the Δ6-desaturases described to date converted for example not only linoleic acid (ω 6-fatty

acid), but also α-linolenic acid (ω3-fatty acid).

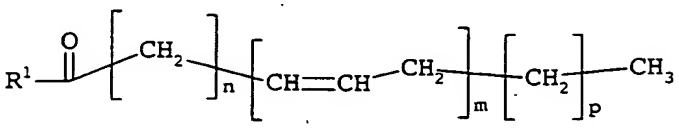
Particularly suitable microorganisms for the production of PUFAs are microorganisms such as Thraustochytrium species or Schizochytrium species, algae such as Phaeodactylum tricornutum or Crypthecodinium species, ciliates such as Stylonychia or Colpidium, fungi such as Mortierella, Entomophthora or Mucor. Strain selection has made possible the development of mutant strains of the microorganisms in question which produce a series of desirable compounds, including PUFAs. The mutation and selection of strains with an improved production of a particular molecule, such as the polyunsaturated fatty acids, is, however, a time-consuming and difficult procedure. This is why recombinant methods are preferred whenever possible, as described above. However, only limited amounts of the desired polyunsaturated fatty acids such as DPA, EPA or ARA can be produced with the aid of the abovementioned microorganisms, these unsaturated fatty acids being obtained, as a rule, as fatty acid mixtures of, for example, EPA, DPA and DHA, depending on the microorganism used.

As an alternative, the production of fine chemicals can suitably be carried out on a large scale via the production in plants which have been developed such that they produce the abovementioned PUFAs. Plants which are particularly suited to this purpose are oil crops, which comprise large amounts of lipid compounds, such as oilseed rape, canola, linseed, soyabeans, sunflowers, borage and evening primrose. However, other crop plants which comprise oils or lipids and fatty acids are also well suited, as mentioned in the extensive description of the present invention. Conventional breeding has given rise to a series of mutant plants which produce a spectrum of desirable lipids and fatty acids, cofactors and enzymes. However, the selection of new plant varieties with improved production of a particular molecule is a time-consuming and difficult procedure or is indeed impossible if the compound does not occur naturally in the plant in question, as in the case of polyunsaturated C_{18}-, C_{20}-fatty acids and C_{22}-fatty acids and those with longer carbon chains.

Owing to the positive properties of unsaturated fatty acids, there has been no lack of attempts in the past to make available these genes which are involved in the synthesis of fatty acids or triglycerides for the production, in various plants, of oils with a modified content of polyunsaturated fatty acids. However, it has been impossible as yet to produce longer-chain polyunsaturated C_{20}- and/or C_{22}-fatty acids such as EPA or ARA in

plants.

It was therefore an object to develop a method for the production of polyunsaturated fatty acid esters and/or free polyunsaturated fatty acids with at least three double bonds in the fatty acid molecule. This object was achieved by the method according to the invention for the production of compounds of the general formula I:



(I)

in transgenic plants with a content of at least 1% by weight based on the total fatty acids, which process comprises the following steps:

a) introducing, into a plant, at least one nucleic acid sequence which encodes a polypeptide with a Δ6-desaturase activity; and

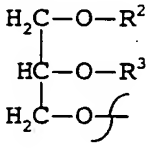
b) introducing at least one second nucleic acid sequence which encodes a polypeptide with a Δ6-elongase activity; and,

c) if appropriate, introducing a third nucleic acid sequence which encodes a polypeptide with a Δ5-desaturase activity;

d) followed by growing and harvesting the plants; and

where the variables and substituents in the formula I have the following meanings:

R^1 = -OH, coenzyme A (thioester), phosphatidylcholine, phosphatidylethanolamine, phoshatidylglycerol, diphosphatidylglycerol, phosphatidylserine, phosphatidylinositol, sphingolipid, glycoshingolipid or a radical of the following general formula II

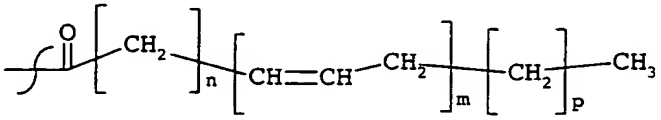


(II)

R^2 = H, phosphatidylcholine-, phosphatidylethanolamine-, phosphatidylglycerol-, diphosphatidylglycerol-, phosphatidylserine-, phosphatidylinositol-, shingolipid-, glycoshingolipid-, glycoshingolipid- or saturated or unsaturated C_2—C_{24}—alkylcarbonyl-,

R^3 = H, saturated or unsaturated C_2—C_{24}—alkylcarbonyl-, or

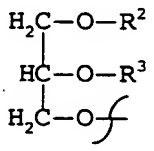
R^2 and R^3 independently of one another represent a radical of the general formula Ia



(Ia),

n = 3, 4 or 6, m = 3, 4 or 5 and p = 0 or 3, preferably n = 3, m = 4 or 5 and p = 0 or 3.

R^1 in the compounds of the formula I denotes -OH (hydroxyl-), acetyl-coenzyme A-, phosphatidylcholine-, phosphatidylethanolamine-, phoshatidylglycerol-, diphosphatidylglycerol-, phosphatidylserine-, phosphatidylinositol-, sphingolipid-, glycoshingolipid- or a radical of the following general formula II



(II)

The abovementioned radicals for R^1 are in each case bound to the compounds of the formula I in the form of esters or thioesters.

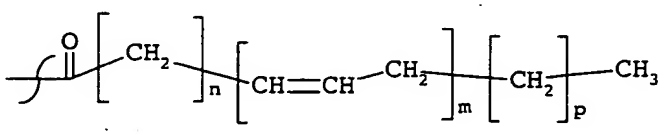
R^2 in the compounds of the formula II denotes hydrogen, phosphatidylcholine-, phosphatidylethanolamine-, phoshatidylglycerol-, diphosphatidylglycerol-, phosphatidylserine-, phosphatidylinositol-, shingolipid-, glycoshingolipid-, glycoshingolipid- or saturated or unsaturated C_2—C_{24}—alkylcarbonyl-.

Unsaturated or saturated C_2-C_{22}-alkylcarbonyl which may be mentioned are radicals such as ethylcarbonyl, n-propylcarbonyl, n-butylcarbonyl, n-pentylcarbonyl, n-hexylcarbonyl, n-heptylcarbonyl, n-octylcarbonyl, n-nonylcarbonyl,

n-decylcarbonyl, n-undecylcarbonyl, n-dodecylcarbonyl, n-tridecylcarbonyl, n-tetradecylcarbonyl, n-pentadecylcarbonyl, n-hexadecylcarbonyl, n-heptadecylcarbonyl, n-octadecylcarbonyl, n-nonadecylcarbonyl, n-eicosylcarbonyl, n-docosanylcarbonyl or n-tetracosanylcarbonyl, all of which may comprise one or more double bonds. Preferred are saturated or unsaturated C_{10}-C_{22}-alkylcarbonyl radicals such as n-decylcarbonyl, n-undecylcarbonyl, n-dodecylcarbonyl, n-tridecylcarbonyl, n-tetradecylcarbonyl, n-pentadecylcarbonyl, n-hexadecylcarbonyl, n-heptadecylcarbonyl, n-octadecylcarbonyl, n-nonadecylcarbonyl, n-eicosylcarbonyl, n-docosanylcarbonyl or n-tetracosanylcarbonyl, all of which comprise one or more double bonds. Especially preferred are saturated or unsaturated C_{10}–C_{22}–alkylcarbonyl radicals such as C_{10}–alkylcarbonyl, C_{11}–alkylcarbonyl, C_{12}–alkylcarbonyl, C_{13}–alkylcarbonyl, C_{14}–alkylcarbonyl, C_{16}–alkylcarbonyl, C_{18}–alkylcarbonyl, C_{20}–alkylcarbonyl, C_{22}–alkylcarbonyl or C_{24}–alkylcarbonyl radicals, all of which comprise one or more double bonds. Very especially preferred are saturated or unsaturated C_{16}–C_{22}–alkylcarbonyl radicals such as C_{16}–alkylcarbonyl, C_{18}–alkylcarbonyl, C_{20}–alkylcarbonyl or C_{22}–alkylcarbonyl radicals, all of which comprise one or more double bonds. Preferably, the abovementioned radicals comprise two, three, four or five double bonds. Especially preferably, the radicals comprise three, four or five double bonds. Very especially preferred are C_{18}–alkylcarbonyl radicals which comprise one, two, three or four double bonds and C_{20}–alkylcarbonyl radicals which comprise three, four or five double bonds. All of the abovementioned radicals are derived from the corresponding fatty acids.

R^3 denotes hydrogen or saturated or unsaturated C_2–C_{24}–alkylcarbonyl.

R^2 and R^3 in the compounds of the formula II independently of one another furthermore denote a radical of the general formula Ia



(Ia),

where n = 3, 4 or 6, m = 3, 4 or 5 and p = 0 or 3, preferably n = 3, m = 4 or 5 and p = 0 or 3.

The abovementioned radicals R^1, R^2 and R^3 may also have attached to them substituents such as hydroxyl or epoxy groups or else comprise triple bonds.

The nucleic acid sequences used in the method according to the invention are isolated nucleic acid sequences which encode polypeptides with Δ5–, Δ6–desaturase or Δ6–elongase activity.

The compounds of the formula I which are produced in this method advantageously comprise a mixture of differing radicals R^1, R^2 or R^3 which can be derived from differing glycerides. Moreover, the abovementioned radicals can be derived from different fatty acids such as short-chain fatty acids having 4 to 6 carbon atoms, medium-chain fatty acids having 8 to 12 carbon atoms or long-chain fatty acids having 14 to 24 carbon atoms; the long-chain fatty acids are preferred.

The method according to the invention advantageously gives fatty acid esters (= compounds of the formula I) with polyunsaturated C_{18}–, C_{20}- and/or C_{22}-fatty acid molecules with at least two double bonds in the fatty acid ester. Preferably, these fatty acid molecules comprise three, four or five double bonds and advantageously lead to the synthesis of γ-linolenic acid (= GLA, $C18:3^{\Delta6,9,12}$), stearidonic acid (= SDA, $C18:4^{\Delta6,9,12,15}$), dihomo-γ-linolenic acid (= DGLA, $20:3^{\Delta8,11,14}$), eicosatetraenoic acid (= ETA, $C20:4^{\Delta5,8,11,14}$), arachidonic acid (ARA), eicosapentaenoic acid (EPA) or their mixtures, preferably EPA and/or ARA.

The fatty acid esters with polyunsaturated C_{18}–, C_{20}- and/or C_{22}-fatty acid molecules can be isolated from the organisms which have been used for the production of the fatty acid esters in the form of an oil or lipid, for example in the form of compounds such as sphingolipids, phosphoglycerides, lipids, glycolipids such as glycoshingolipid, phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phoshatidylserine, phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol, monoacylglycerides, diacylglycerides, triacylglycerides or other fatty acid esters such as the acetyl-coenzyme A esters which comprise the polyunsaturated fatty acids having at least two, preferably three, double bonds. In addition to these esters, the polyunsaturated fatty acids are also present in the plants as free fatty acids or bound in other compounds. As a rule, the different abovementioned compounds (fatty acid esters and free fatty acids) are present in the plant in an approximate distribution of 80 to 90% by weight of triglycerides, 2 to 5% by weight of diglycerides, 5 to 10% by

weight of monoglycerides, 1 to 5% by weight of free fatty acids, 2 to 8% by weight of phospholipids, the total of the different compounds making 100% by weight.

When the compounds of the general formula I are produced in the method according to the invention, they are produced in a content of at least 1% by weight, advantageously at least 2% by weight, preferably at least 3% by weight, especially preferably at least 5% by weight, very especially preferably at least 10% by weight based on the total of the fatty acids in the transgenic plant. Since, in the method according to the invention, the starting compounds linoleic acid (C18:2) and/or linolenic acid (C18:3) undergo several reaction steps, the end products of the method, such as, for example, arachidonic acid (ARA) or eicosapentaenoic acid (EPA) are not obtained as pure products, but there are always minor amounts of the precursors still present in the end product. If both linoleic acid and linolenic acid are present in the original plant, the end products such as ARA and EPA are present as mixtures. The precursors should advantageously not amount to more than 20% by weight, preferably not more than 15% by weight, especially preferably not more than 10% by weight, very especially preferably not more than 5% by weight, based on the amount of the end product in question. Advantageously, the end products which are produced in the method according to the invention in a transgenic plant are only ARA or only EPA, either bound or as free acids (see compounds of the general formula I). If both compounds (ARA + EPA) are produced simultaneously, they are advantageously prduced in a ratio of at least 1:2 (EPA:ARA), advantageously at least 1:3, preferably 1:4, especially preferably 1:5.

Suitable organisms for the production in the method according to the invention are, in principle, all plants such as mosses, algae, dicots or monocots. It is advantageous to use, in the method according to the invention, organisms which belong to the oil-producing organisms, i.e. which are used for the production of oils, such as algae like Crypthecodinium, Phaeodactylum or plants, in particular plants, preferably oil crops, which comprise large amounts of lipid compounds, such as peanut, oilseed rape, canola, sunflower, safflower, poppy, mustard, hemp, castor-oil plant, olive, sesame, Calendula, Punica, evening primrose, verbascum, thistle, wild roses, hazelnut, almond, macadamia, avocado, bay, pumpkin/squash, linseed, soybean, pistachios, borage, trees (oil palm, coconut or walnut) or field crops such as maize, wheat, rye, oats, triticale, rice, barley, cotton, cassava, pepper, Tagetes, Solanaceae plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa

or bush plants (coffee, cacao, tea), Salix species and perennial grasses and fodder crops. Preferred plants according to the invention are oil crops such as peanut, oilseed rape, canola, sunflower, safflower, pea, mustard, hemp, castor-oil plants, olive, Calendula, Punica, evening primrose, pumpkin/squash, linseed, soybean, borage, trees (oil palm, coconut). Especially preferred are plants which are high in C18:2- and/or C18:3-fatty acid, such as sunflower, safflower, tobacco, verbascum, sesame, cotton, pumpkin/squash, poppy, evening primrose, walnut, linseed, hemp, thistle or safflower. Very especially preferred are plants such as safflower, sunflower, poppy, evening primrose, walnut, linseed or hemp.

Owing to the enzymatic activity of the nucleic acids used in the method according to the invention, which encode polypeptides with Δ5-, Δ6-desaturase or Δ6-elongase activity, different compounds of the formula I can be produced. Depending on the choice of the plant used for the method according to the invention, mixtures of the different compounds of the general formula I or individual compounds, such as EPA or ARA, can be produced in free or bound form. Depending on the fatty acid composition which prevails in the original plant (C18:2- or C18:3-fatty acids), this gives compounds of the general formula I which are derived from C18:2-fatty acids, such as GLA-, DGLA- or ARA-comprising compounds of the formula I, or compounds which are derived from C18:3-fatty acids, such as SDA-, ETA- or EPA-comprising compounds of the formula I. If linoleic acid (= LA, C18:$2^{\Delta 9,12}$) is the only unsaturated fatty acid present in the plant used for the method, only GLA, DGLA and ARA can be formed as products of the method, all of which can be present as free fatty acids or in bound form. If α-linolenic acid (= ALA, C18:$3^{\Delta 9,12,15}$) is the only unsaturated fatty acid present in the plant used in the method, for example such as in linseed, only SDA, ETA and EPA can be formed as products of the method, all of which can be present as free fatty acids or in bound form, as described above. By modifying the activity of the enzymes implicated in the synthesis (Δ5-, Δ6-desaturase and Δ6-elongase), or by introducing only the first two genes (Δ6-desaturase and Δ6-elongase) of the synthetic cascade, it is possible to produce in a targeted manner only individual products in the abovementioned plants (see Figure I). Due to the activity of the enzymes Δ6-desaturase and Δ6-elongase, GLA and DGLA, or SDA and ETA, respectively, form, depending on the original plant and the unsaturated fatty acid. DGLA or ETA, respectively, or mixtures of these are formed preferentially. If the enzyme Δ5-desaturase is additionally introduced into the plant, ARA or EPA are additionally formed. It is advantageous only to synthesize ARA or EPA or their mixtures, depending on the

fatty acid which is present in the plant and which acts as starting material for the synthesis. Since biosynthetic cascades are involved, the end products in question are not present in pure form in the plants. There are always minor amounts of the precursor compounds present in the end product. These minor amounts amount to less than 20% by weight, advantageously less than 15% by weight, especially advantageously less than 10% by weight, very especially advantageously less than 5, 4, 3, 2 or 1% by weight, based on the end product DGLA, ETA or their mixtures, or ARA, EPA or their mixtures, respectively.

For the purposes of the method according to the invention, transgenic plants are also understood as meaning plant cells, plant organs or intact plants which are grown for the production of compounds of the general formula I. Growing is understood as meaning for example culturing of the transgenic plant cells, plant tissue or plant organs on a nutrient medium or the intact plant on or in a substrate, for example in hydroponic culture or on an arable soil.

Nucleic acids which can be used in the method according to the invention are, in principle, all those which encode polypeptides with Δ5—, Δ6—desaturase- or Δ6—elongase activity. These nucleic acids are advantageously derived from plants such as algae, such as Isochrysis or Crypthecodinium, diatoms such as Phaeodactylum, mosses such as Physcomitrella, Ceratodon or higher plants such as the primulaceae, such as Aleuritia, Calendula stellata, Osteospermum spinescens or Osteospermum hyoseroides, microorganisms such as fungi, such as Aspergillus, Thraustochytrium, Phytophtora, Entomophthora, Mucor or Mortierella, yeasts or animals such as nematodes, such as Caenorhabditis, insects or humans. The Δ5—, Δ6—desaturase or Δ6—elongase genes are advantageously derived from fungi or from plants such as algae or mosses, preferably from plants.

It is advantageous to in the method according to the invention, a nucleic acid sequence selected from the group of the in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 or their derivative or homologs which encode polypeptides which retain the enzymatic activity. These sequences, individually or in combination, are cloned into expression constructs; these expression constructs are represented in the sequences SEQ ID NO: 33-37. These expression constructs make possible an optimal synthesis of the compounds of the general formula I produced in the method according to the invention.

In a preferred embodiment, the method furthermore comprises the step of obtaining a cell which comprises the nucleic acid sequences which are used in the method and which encode a Δ5– or Δ6–desaturase and a Δ6–elongase, where a cell is transformed with the nucleic acid sequence, a gene construct or a vector which bring about the expression of the Δ5–, Δ6–desaturase or Δ6–elongase nucleic acid, alone or in combination. In a further preferred embodiment, the method furthermore comprises the step of obtaining the fine chemical from the culture. The cell generated thus is advantageously a cell of an oil crop such as, for example, peanut, oilseed rape, canola, linseed, soybean, safflower, hemp, sunflowers or borage.

A transgenic plant is understood as meaning, for the purposes of the invention, that the nucleic acids used in the method are not at their natural locus in the genome of an organism; in this context, the nucleic acids can be expressed homologously or heterologously. However, transgenic also means that, while the nucleic acids according to the invention are at their natural locus in the genome of an organism, the sequence has been modified in comparison with the natural sequence and/or the regulatory sequences of the natural sequences have been modified. Preferably, transgenic is understood as meaning that the nucleic acids according to the invention are not expressed at their natural locus in the genome, that is to say that homologous or preferably heterologous expression of the nucleic acids takes place. Preferred transgenic plants are the oil crops.

Transgenic plants which comprise the compounds of the formula I which have been synthesized in the method according to the invention can be marketed directly without isolation of the compounds which have been synthesized. Plants are understood as meaning, in the method according to the invention, all plant parts, plant organs such as leaf, stem, root, tuber or seeds, or all of the plant. In this context, the seed comprises all parts of the seed such as the seed coats, epidermis cells and seed cells, endosperm or embyro tissue. However, the compounds produced in the method according to the invention can also be isolated from the plants in the form of their oils, fat, lipids and/or free fatty acids. Compounds of the formula I which have been produced by this method can be harvested by harvesting the organisms either from the culture in which they grow or from the field. This can be done by pressing or extracting the plant parts, preferably the plant seeds. In this context, the oils, fats, lipids and/or free fatty acids can be obtained by pressing by what is known as cold-beating or cold-pressing, without supplying heat. The plant parts, specifically the seeds, are

beforehand comminuted, steam-treated or toasted in order to facilitate their disruption. The seeds pretreated thus can subsequently be pressed or else extracted with solvents such as warm hexane. The solvent is subsequently removed. In this manner, more than 96% of the compounds produced in the method can be isolated. The resulting products are subsequently processed further, i.e. refined. Here, the plant mucilages and turbid matter are first. What is known as degumming can be performed enzymatically or, for example, chemico-physically by adding acid such as phosphoric acid. The free fatty acids are subsequently removed by treatment with a base, for example sodium hydroxide solution. The resulting product is washed thoroughly with water to remove the alkali remaining in the product, and dried. To remove the coloring matter which still remains in the product, the products are bleached, for example using bleaching earth or active charcoal. At the end, the product is deodorized, for example by using steam.

The PUFAs produced by this method are preferentially C_{18}- or C_{20-22}-fatty acid molecules having at least two double bonds in the fatty acid molecule, preferably three, four, in combination with a further elongases and a Δ4-desaturase five or six double bonds. These C_{18}- or C_{20-22}-fatty acid molecules can be isolated from the organism in the form of an oil, lipid or a free fatty acid. Suitable organisms are, for example, those which have been mentioned above. Preferred organisms are transgenic plants.

In a preferred embodiment, oils, lipids or fatty acids or fractions of these which have been produced by the above-described method are especially preferably oil, lipid or a fatty acid composition which comprise PUFAs or which originate from transgenic plants.

A further embodiment according to the invention is the use of the oil, lipid or the fatty acid composition in foods, feeds, cosmetics or pharmaceuticals.

The term "oil" or "fat" is understood as meaning a fatty acid mixture which comprises unsaturated, saturated, preferably esterified fatty acid(s). It is preferred that the oil or fat has a high content of unsaturated, unconjugated esterified fatty acid(s), in particular linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, α-linolenic acid, stearidonic acid, eicosatetraenoic acid or eicosapentaenoic acid. The amount of unsaturated esterified fatty acids is preferably approximately 30%, with an amount of 50% being more preferred and an amount of 60%, 70%, 80% or more being even more preferred. For

identification purposes, it is possible, for example, to determine the amount of fatty acid by gas chromatography after converting the fatty acids into the methyl esters by means of transesterification. The oil or fat can comprise various other saturated or unsaturated fatty acids, for example calendulic acid, palmitic acid, stearic acid, oleic acid and the like. The amount of the various fatty acids in oil or fat can vary in particular as a function of the original plant.

The compounds of the formula I which are produced in the method and which comprise polyunsaturated fatty acids having at least two double bonds are sphingolipids, phosphoglycerides, lipids, glycolipids, phospholipids, monoacylglycerol, diacylglycerol, triacylglycerol or other fatty acid esters.

The polyunsaturated fatty acids which are present can be liberated from the compounds of the general formula I produced thus in the method according to the invention for example via treatment with alkali, for example aqueous KOH or NaOH, or acid hydrolysis, advantageously in the presence of an alcohol such as methanol or ethanol, or via enzymatic cleavage and isolated via, for example, phase separation and subsequent acidification with, for example, H_2SO_4. However, the fatty acids can also be liberated directly without the above-described processing.

After they have been introduced into plant cells or plants, the nucleic acids used in the method can either be located on a separate plasmid or integrated into the genome of the host cell. In the case of integration into the genome, the integration can be random or be effected by recombination in such a way that the native gene is replaced by the copy being introduced, whereby the production of the desired compound by the cell is modulated, or by using a gene in trans, so that the gene is linked operably with a functional expression unit which comprises at least one sequence which ensures the expression of a gene and at least one sequence which ensures the polyadenylation of a functionally transcribed gene. The nucleic acids are advantageously introduced into the plants via multiexpression cassettes or constructs for the multiparallel seed-specific expression of genes.

Mosses and algae are the only known plant systems which produce substantial amounts of polyunsaturated fatty acids such as arachidonic acid (ARA) and/or eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA). Mosses comprise PUFAs in membrane lipids, while algae, organisms which are related to algae and some fungi also accumulate substantial amounts of PUFAs in the triacylglycerol fraction. This is why nucleic acid molecules

which are isolated from such strains which also accumulate PUFAs in the triacylglycerol fraction are especially advantageously suitable for the method according to the invention and thus for the modification of the lipid and PUFA production system in a host, in particular plants, such as oil crops, for example oilseed rape, canola, linseed, hemp, soybean, sunflowers, borage. They can therefore be used advantageously in the method according to the invention.

It has been possible to date to demonstrate that a trienoic acid with C_{18} carbon chain can be produced with the aid of desaturases. These methods which are known from the literature claim the production of γ-linolenic acid. However, nobody has as yet been able to demonstrate the production very long-chain polyunsaturated fatty acids (with C_{20}- and longer carbon chain and of trienoic acids and higher unsaturated types) by modified plants alone.

To produce the longer-chain PUFAs according to the invention, the polyunsaturated C_{18}-fatty acids must first be desaturated by the enzymatic activity of a desaturase and subsequently elongated by at least two carbon atoms via an elongase. After one elongation cycle, this enzyme activity gives C_{20}-fatty acids, and after two or three elongation cycles C_{22}- or C_{24}-fatty acids. The activity of the desaturases and elongases used method according to the invention gives by preference C_{18}-, C_{20}- and/or C_{22}-fatty acids having at least two double bonds in the fatty acid molecule, by preference three, four or five double bonds, especially preferably C_{18}- and/or C_{20}-fatty acids with at least two double bonds in the fatty acid molecule, prefereably with three, four or five double bonds in the molecule. After a first desaturation and the elongation have taken place, further desaturation steps such as, for example, in Δ5-position, may take place. Especially preferred products of the process according to the invention are arachidonic acid and eicosapentaenoic acid. The C_{18}-fatty acids with at least two double bonds in the fatty acid can be elongated by the enzymatic activity according to the invention in the form of the free fatty acid or in the form of the esters, such as phospholipids, glycolipids, sphingolipids, phosphoglycerides, monoacylglycerol, diacylglycerol or triacylglycerol.

Using cloning vectors in plants and in the transformation of plants like those which are published and cited in: Plant Molecular Biology and Biotechnology (CRC Press, Boca Raton, Florida), Chapter 6/7, pp. 71-119 (1993); F.F. White, Vectors for Gene Transfer in Higher Plants; in: Transgenic Plants, Vol. 1, Engineering and Utilization, Eds.: Kung and R. Wu, Academic

Press, 1993, 15-38; B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, Eds.: Kung and R. Wu, Academic Press (1993), 128-143; Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225)), the nucleic acids can be used for the recombinant modification of a broad spectrum of plants so that this plant becomes a better or more efficient producer of one or more lipid-derived products, such as PUFAs. This improved production or production efficiency of a lipid-derived product, such as PUFAs, can be brought about by a direct action of the manipulation or an indirect action of this manipulation.

A series of mechanisms exist by means of which the modification of a desaturase protein according to the invention can have a direct effect on the yield, production and/or production efficiency of a fine chemical from an oil crop plant or a microorganism, owing to a modified protein. The number or activity of the desaturase protein or desaturase gene and of gene combinations of desaturases and elongases can be increased, so that larger amounts of these compounds are produced de novo since the organisms lacked this activity and ability to biosynthesize them prior to introduction of the gene in question. This also applies analogously to the combination with further desaturases or elongases or further enzymes of the lipid metabolism. The use of various divergent sequences, i.e. sequences which differ at the DNA sequence level, may also be advantageous, or else the use of promoters for gene expression which makes possible a different temporal gene expression, for example as a function of the degree of maturity of the seed or oil-storing tissue.

The introduction of a desaturase and/or elongase gene, or several desaturase and elongase genes, into an organism, alone or in combination with other genes into a cell can not only increase the biosynthesis flux toward the end product, but also increase, or generate de novo, the corresponding triacylglycerol composition. Likewise, the number or activity of other genes which participate in the import of nutrients required for the biosynthesis of one or more fine chemicals (for example fatty acids, polar and neutral lipids) can be increased, so that the concentration of these precursors, cofactors or intermediates within the cells or within the storage compartment is increased, thus further increasing the ability of the cells to produce PUFAs as described hereinbelow. Fatty acids and lipids themselves are desirable as fine chemicals; by optimizing the activity or increasing the number of one or more desaturases and/or elongases which participate in the biosynthesis of these compounds, or by destroying the activity of one or more desaturases which

participate in the breakdown of these compounds, it can be possible to increase the yield, production and/or efficiency of the production of fatty acid and lipid molecules from plants.

The isolated nucleic acid molecules used in the process according to the invention encode proteins or parts of these, the proteins, or the individual protein or parts thereof, comprising an amino acid sequence with sufficient homology with an amino acid sequence of the sequence SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32 so that the protein or the part thereof retains a desaturase or elongase activity. Preferably, the protein or the part thereof which is encoded by the nucleic acid molecule has its essential enzymatic activity and the capability of being implicated in the metabolism of compounds which are required for the synthesis of plant cell membranes or in the transport of molecules across these membranes. Advantageously, the protein encoded by the nucleic acid molecules is at least approximately 50%, preferably at least approximately 60% and more preferably at least approximately 70%, 80% or 90% and most preferably at least approximately 95%, 96%, 97%, 98%, 99% or more homologous to an amino acid sequence of the sequence SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32. Preferably, the protein is a full-length protein which is essentially homologous in parts to a total amino acid sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32 (which is the result of the open reading frame shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31). For the purposes of the invention, homology and homologous are understood as meaning identity or identical.

The term essential enzymatic activity of the desaturases and the elongase used is understood as meaning that, in comparison with the proteins/enzymes encoded by the sequences with SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31, they retain at least an enzymatic activity of at least 10%, preferably 20%, especially preferably 30% and very especially 40% and can thus be implicated in the metabolism of compounds which are required for the synthesis of fatty acids in a plant cell or in the transport of molecules across membranes, meaning desaturated C_{18}- or C_{20-22}- carbon chains with double bonds at at least two, advantageoulsy three, four or five positions.

Nucleic acids which can advantageously be used in the process originate from fungi or plants such as algae or mosses of the genera Physcomitrella, Thraustochytrium, Phytophtora, Ceratodon, Isochrysis, Aleurita, Muscarioides, Mortierella, Borago, Phaeodactylum, Crypthecodinium or from nematodes such as

Ceanorhabditis, specifically from the genera and species Physcomitrella patens, Phytophtora infestans, Ceratodon purpureus, Isochrysis galbana, Aleurita farinosa, Muscarioides viallii, Mortierella alpina, Borago officinalis, Phaeodactylum tricormutum or Ceanorhabditis elegans.

As an alternative, the isolated nucleotide sequences used can encode desaturases or elongases which hybridize, for example under stringent conditions, with a nucleotide sequence of the SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31.

The nucleic acid sequences used in the process are advantageously introduced in an expression cassette which makes possible the expression of the nucleic acids in plants.

Advantageous expression cassettes are shown in SEQ ID NO: 33 to 37. Here, the nucleic acid sequences encoding the desaturases and/or the elongases are linked operably with one or more regulatory signals, advantageously for enhancing gene expression. These regulatory sequences are intended to make possible the specific expression of genes and of protein expression. Depending on the host organism, this may mean, for example, that the gene is expressed and/or overexpressed only after induction, or else that it is immediately expressed and/or overexpressed. For example, these regulatory sequences take the form of sequences to which inductors or repressors bind and thus regulate expression of the nucleic acid. In addition to these novel regulatory sequences, or instead of these sequences, the natural regulation of these sequences before the actual structural genes may still be present and, if appropriate, may have been genetically modified so that the natural regulation has been switched off and the expression of the genes enhanced. However, the expression cassette (= expression construct = gene construct) can also be simpler in construction, that is to say no additional regulatory signals have been inserted before the nucleic acid sequence or its derivatives, and the natural promoter together with its regulation has not been removed. Instead, the natural regulatory sequence has been mutated in such a way that regulation no longer takes place and/or gene expression is enhanced. These modified promoters can also be placed before the natural gene alone in the form of part-sequences (= promoter together with parts of the nucleic acid sequences according to the invention) to enhance the activity. Moreover, the gene construct can advantageously also comprise one or more enhancer sequences in operable linkage with the promoter, which make possible an enhanced expression of the nucleic acid sequence. Also, additional advantageous sequences,

such as further regulatory elements or terminators, may be inserted at the 3' terminus of the DNA sequences. The Δ5-desaturase/Δ6-desaturase and/or Δ6-elongase genes may be present in the expression cassette (= gene construct) in one or more copies. Advantageously, in each case only one copy of the genes is present in the expression cassette. This gene construct, or the gene constructs, can be expressed together in the host organism. In this context, the gene construct(s) can be inserted in one or more vectors and be present in the cell in free form or else be inserted in the genome. It is advantageous for the insertion of further genes in the host genome when the genes to be expressed are present together in one gene construct.

In this context, the regulatory sequences or factors can, as described above, preferably have a positive effect on the gene expression of the genes which have been introduced, thus enhancing it. Thus, the regulatory elements can advantageously be enhanced at transcriptional level by using strong transcription signals such as promoters and/or enhancers. In addition, however, an enhancement of translation is also possible, for example by improving the stability of the mRNA.

A further embodiment of the invention are one or more gene constructs which comprise one or more sequences which are defined by SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 and which encode polypeptides of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32. The abovementioned desaturases introduce a double bond into the Δ5 or Δ6 position, the substrate having one, two, three or four double bonds. Elongase (Δ6-elongase) has an enzyme activity which elongates a fatty acid by at least two carbon atoms. The same applies to its homologs, derivatives or analogs which are linked operably with one or more regulatory signals, advantageously for enhancing gene expression.

Advantageous regulatory sequences for the novel process are present, for example, in promoters such as cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, $lacI^q$, T7, T5, T3, gal, trc, ara, SP6, λ-P_R or λ-P_L promoter and are advantageously used in Gram-negative bacteria. Further advantageous regulatory sequences are present, for example, in the Gram-positive promoters amy and SPO2, in the yeast or fungal promoters ADC1, MFα, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH or in the plant promoters CaMV/35S [Franck et al., Cell 21 (1980) 285-294], PRP1 [Ward et al., Plant. Mol. Biol. 22 (1993)], SSU, OCS, lib4, usp, STLS1, B33, nos or in the ubiquitin or phaseolin promoter. Also advantageous in this connection are inducible promoters such as the promoters described in EP-A-0

388 186 (benzylsulfonamide-inducible), Plant J. 2, 1992:397–404 (Gatz et al., tetracyclin-inducible), EP–A–0 335 528 (abscisic acid-inducible) or WO 93/21334 (ethanol- or cyclohexenol-inducible). Further useful plant promoters are the potato cytosolic FBPase promoter or ST-LSI promoter (Stockhaus et al., EMBO J. 8, 1989, 2445), the Glycine max phosphoribosyl-pyrophosphate amidotransferase promoter (Genbank Accession No. U87999) or the node-specific promoter described in EP–A–0 249 676. Especially advantageous promoters are promoters which make possible expression in tissues which are implicated in fatty acid biosynthesis. Very especially advantageous are seed-specific promoters, such as the USP promoter in accordance with the specification, but also other promoters such as the LeB4, DC3, phaseolin or napin promoter. Further especially advantageous promoters are seed-specific promoters which can be used for monocots or dicots and which are described in US 5,608,152 (oilseed rape napin promoter), WO 98/45461 (Arabidopsis oleosin promotor), US 5,504,200 (Phaseolus vulgaris phaseolin promoter), WO 91/13980 (Brassica Bce4 promoter) described by Baeumlein et al., Plant J., 2, 2, 1992:233–239 (LeB4 promoter from a legume), said promoters being useful in dicots. The following promoters are suitable for example in monocots: barley lpt–2 or lpt–1 promoter (WO 95/15389 and WO 95/23230), barley hordein promoter and other suitable promoters which are described in WO 99/16890.

In principle, it is possible to use all natural promoters with their regulatory sequences like those mentioned above for the novel process. It is likewise possible and advantageous to use synthetic promoters, in addition or alone, especially when they confer seed-specific expression, such as, for example, described in WO 99/16890.

In order to achieve a particularly high PUFA content in transgenic plants, the PUFA biosynthetic genes should advantageously be expressed in oil crops in a seed-specific manner. To this end, seed-specific promoters can be used, or those promoters which are active in the embryo and/or in the endosperm. In principle, seed-specific promoters can be isolated from both dicots and monocots. Advantageous preferred promoters are detailed hereinbelow: USP (= unknown seed protein) and vicilin (Vicia faba) [Bäumlein et al., Mol. Gen Genet., 1991, 225(3)], napin (oilseed rape) [US 5,608,152], Acyl-Carrier Protein (oilseed rape) [US 5,315,001 and WO 92/18634], oleosin (Arabidopsis thaliana) [WO 98/45461 and WO 93/20216], phaseolin (Phaseolus vulgaris) [US 5,504,200], Bce4 [WO 91/13980], legume B4 (LegB4 promoter) [Bäumlein et al., Plant J., 2, 2, 1992], Lpt2 and lpt1 (barley) [WO 95/15389 and WO95/23230], seed-specific

promoters from rice, maize and wheat [WO 99/16890], Amy32b, Amy 6-6 and aleurain [US 5,677,474], Bce4 (oilseed rape) [US 5,530,149], glycinin (soya) [EP 571 741], phosphoenolpyruvate carboxylase (soya) [JP 06/62870], ADR12-2 (soya) [WO 98/08962], isocitrate lyase (oilseed rape) [US 5,689,040] or β-amylase (barley) [EP 781 849].

Plant gene expression can also be facilitated via a chemically inducible promoter (see a review in Gatz 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48:89-108). Chemically inducible promoters are particularly suitable when it is desired that gene expression should take place in a time-specific manner. Examples of such promoters are a salicylic-acid-inducible promoter (WO 95/19443), a tetracyclin-inducible promoter (Gatz et al. (1992) Plant J. 2, 397-404) and an ethanol-inducible promoter.

To ensure the stable integration of the biosynthesis genes into the transgenic plant over a plurality of generations, each of the nucleic acids which encode Δ6-desaturase, Δ5-desaturase, or Δ6-elongase and which are used in the process should be expressed under the control of a separate promoter, preferably a promoter which differs from the other promoters, since repeating sequence motifs can lead to instability of the T-DNA, or to recombination events. In this context, the expression cassette is advantageously constructed in such a way that a promoter is followed by a suitable cleavage site, advantageously in a polylinker, for insertion of the nucleic acid to be expressed and, if appropriate, a terminator sequence is positioned behind the polylinker. This sequence is repeated several times, preferably three, four or five times, so that up to five genes can be combined in one construct and introduced into the transgenic plant in order to be expressed. Advantageously, the sequence is repeated up to three times (see sequence listing SEQ ID NO: 33 to 37). To express the nucleic acid sequences, the latter are inserted after the promoter via a suitable cleavage site, for example in the polylinker. Advantageously, each nucleic acid sequence has its own promoter and, if appropriate, its own terminator sequence. However, it is also possible to insert a plurality of nucleic acid sequences after a promoter and, if appropriate, before a terminator sequence. Here, the insertion site, or the sequence, of the inserted nucleic acids in the expression cassette is not of critical importance, that is to say a nucleic acid sequence can be inserted at the first or last position in the cassette without its expression being substantially influenced thereby. Advantageously, different promoters such as, for example, the USP, LegB4 or DC3 promoter, and different terminator sequences can be used in the expression

cassette. However, it is also possible to use only one type of promoter in the cassette, which, however, may lead to undesired recombination events.

As described above, the transcription of the genes which have been introduced should advantageously be terminated by suitable terminator sequences at the 3' end of the biosynthesic genes which have been introduced (after the stop codon). An example of a sequence which can be used in this context is the OCS1 terminator sequence. As is the case with the promoters, different terminator sequences should be used for each gene.

As described above, the gene construct can also comprise further genes to be introduced into the organisms. It is possible and advantageous to introduce into the host organisms, and to express, regulatory genes such as genes for inductors, repressors or enzymes which, owing to their enzyme activity, engage in the regulation of one or more genes of a biosynthesis pathway. These genes can be of heterologous or of homologous origin. Moreover, further biosynthesis genes of the fatty acid or lipid metabolism can advantageously be present in the nucleic acid construct, or gene construct; however, these genes can also be present on one or more further nucleic acid constructs. A biosynthesic gene of the fatty acid or lipid metabolism which is preferably chosen is a gene selected from the group acyl-CoA dehydrogenase(s), acyl-ACP [= acyl carrier protein] desaturase(s), acyl—ACP thioesterase(s), fatty acid acyltransferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme A carboxylase(s), acyl—coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid acetylenases, lipoxygenases, triacylglycerol lipases, allene oxide synthases, hydroperoxide lyases or fatty acid elongase(s) or their combinations.

In this context, the abovementioned desaturases can be cloned into expression cassette according to the invention in combination with elongases and other desaturases and employed for the transformation of plants with the aid of Agrobacterium.

In this context, the regulatory sequences or factors can, as described above, have a positive effect on, preferably, the gene expression of the genes introduced, thus enhancing it. Thus, enhancement of the regulatory elements can advantageously take place at the transcriptional level by using strong transcription signals such as promoters and/or enhancers. In addition, however, enhancement of translation is also possible, for example by improving the stability of the mRNA. In principle, the expression

cassettes can be used directly for introduction into the plant, or else be introduced into a vectors.

These advantageous vectors, preferably expression vectors, comprise the nucleic acid which are used in the method and which encode Δ5- or Δ6-desatures or Δ6-elonagases, or a nucleic acid construct, which the nucleic acid used, alone or in combination with further biosynthetic genes of the fatty acid or lipid metabolism. As used in the present context, the term "vector" refers to a nucleic acid molecule which is capable of transporting another nucleic acid, to which it is bound. One type of vector is a "plasmid", which represents a circular double-stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, it being possible for additional DNA segments to be ligated in the viral genome. Certain vectors are capable of autonomous replication in a host cell in which they have been introduced (for example bacterial vectors with bacterial origin of replication). Other vectors are advantageously integrated in the genome of a host cell when being introduced into the host cell, whereby they replicate together with the host genome. Moreover, certain vectors are capable of governing the expression of genes with which they are operably linked. These vectors are referred to herein as "expression vectors". Usually, expression vectors which are suitable for DNA recombination techniques take the form of plasmids. In the present description, "plasmid" and "vector" can be used interchangeably since the plasmid is the most frequently used vector form. However the invention is also intended to comprise these other forms of expression vectors, such as viral vectors, which have similar functions. Furthermore, the term vector is also intended to comprise other vectors which are known to the skilled worker, such as phages, viruses such as SV40, CMV, TMV, transposons, IS elements, phasmids, phagemids, cosmids, linear or circular DNA.

The recombinant expression vectors which are advantageously used in the method comprise the nucleic acids described hereinbelow or the above-described gene construct in a form suitable for expressing these nucleic acids in a host cell, which means that the recombinant expression vectors comprise one or more regulatory sequences selected on the basis of the host cells to be used for the expression, which is linked operably with the nucleic acid sequence to be expressed. "Linked operably" in a recombinant expression vector means that the nucleotide sequence of interest is bound to the regulatory sequence(s) in such a way that the expression of the nucleotide sequence is possible and that they are bound with one another so that both sequences

fulfill the predicted function ascribed to the sequence (for example in an in-vitro transcription/translation system or in a host cell if the vector is introduced into the host cell). The term "regulatory sequence" is intended to comprise promoters, enhancers and other expression control elements (for example polyadenylation signals). These regulatory sequences are described for example in Goeddel: Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990), or see: Gruber and Crosby, in: Methods in Plant Molecular Biology and Biotechnolgy, CRC Press, Boca Raton, Florida, eds.: Glick and Thompson, chapter 7, 89-108, including the references therein. Regulatory sequences comprise those which govern the constitutive expression of a nucleotide sequence in many types of host cell and those which govern the direct expression of the nucleotide sequence only in specific host cells under specific conditions. The skilled worker knows that the design of the expression vector can depend on factors such as the choice of the host cell to be transformed, the expression level of the desired protein and the like.

The recombinant expression vectors used can be designed for expressing desaturases and elongases in prokaryotic or eukaryotic cells. This is advantageous since intermediate steps of vector construction are frequently performed in microorganisms for the sake of simplicity. For example, desaturase and/or elongase genes can be expressed in bacterial cells, insect cells (using baculovirus expression vectors), yeast cells and other fungal cells (see Romanos, M.A., et al. (1992) "Foreign gene expression in yeast: a review", Yeast 8:423-488; van den Hondel, C.A.M.J.J., et al. (1991) "Heterologous gene expression in filamentous fungi", in: More Gene Manipulations in Fungi, J.W. Bennet & L.L. Lasure, ed., pp. 396-428: Academic Press: San Diego; and van den Hondel, C.A.M.J.J., & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: Applied Molecular Genetics of Fungi, Peberdy, J.F., et al., ed., pp. 1-28, Cambridge University Press: Cambridge), Algen (Falciatore et al., 1999, Marine Biotechnology. 1, 3:239-251), ciliates of the types: Holotrichia, Peritrichia, Spirotrichia, Suctoria, Tetrahymena, Paramecium, Colpidium, Glaucoma, Platyophrya, Potomacus, Desaturaseudocohnilembus, Euplotes, Engelmaniella and Stylonychia, in particular the genus Stylonychia lemnae, using vectors by a transformation method as described in WO 98/01572, and preferably in cells of multi-celled plants (see Schmidt, R. and Willmitzer, L. (1988) "High efficiency *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants" Plant Cell Rep.:583-586; Plant Molecular Biology and Biotechnology, C Press, Boca Raton, Florida, chapter

6/7, pp.71-119 (1993); F.F. White, B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, vol. 1, Engineering and Utilization, ed.: Kung and R. Wu, Academic Press (1993), 128-43; Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225 (and references cited therein)). Suitable host cells are furthermore discussed in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). As an alternative, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulation sequences and T7 polymerase.

Protein expression in prokaryotes is usually performed with the aid of vectors which comprise constitutive or inducible promoters which govern the expression of fusion proteins or nonfusion proteins. Typical fusion expression vectors are, inter alia pGEX (Pharmacia Biotech Inc; Smith, D.B., and Johnson, K.S. (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ), where glutathione S-transferase (GST), maltose-E-binding protein or protein A, respectively, is fused with the recombinant target protein.

Examples of suitable inducible nonfusion E. coli expression vectors are, inter alia, pTrc (Amann et al. (1988) Gene 69:301-315) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 60-89). The target gene expression of the pTrc vector is based on the transcription of host RNA polymerase by a hybrid trp-lac fusion promoter. The target gene expression from the pET 11d vector is based on the transcription of a T7-gn10-lac fusion promoter, which is mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is provided by the host strains BL21 (DE3) or HMS174 (DE3) by a resident λ prophage which harbors a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

Other vectors which are suitable for use in prokaryotic organisms are known to the skilled worker; these vectors are, for example in E. coli, pLG338, pACYC184, the pBR series such as pBR322, the pUC series such as pUC18 or pUC19, the M113mp series, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III^{113}-B1, λgt11 or pBdCI, in Streptomyces pIJ101, pIJ364, pIJ702 or pIJ361, in Bacillus pUB110, pC194 oder pBD214, in Corynebacterium pSA77 or pAJ667.

In a further embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in the yeast S. cerevisiae comprise pYeDesaturasec1 (Baldari et al.

(1987) Embo J. 6:229-234), pMFa (Kurjan and Herskowitz (1982) Cell 30:933-943), pJRY88 (Schultz et al. (1987) Gene 54:113-123) and pYES2 (Invitrogen Corporation, San Diego, CA). Vectors and methods for construction of vectors which are suitable for use in other fungi, such as the filamentous fungi, comprise those which are described in detail in: van den Hondel, C.A.M.J.J., & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: Applied Molecular Genetics of fungi, J.F. Peberdy et al., ed., pp. 1-28, Cambridge University Press: Cambridge, or in: More Gene Manipulations in Fungi [J.W. Bennet & L.L. Lasure, ed., pp. 396-428: Academic Press: San Diego]. Further suitable yeast vectors are, for example, pAG-1, YEp6, YEp13 or pEMBLYe23.

As an alternative, the desaturases and/or elongases can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors which are available for expressing proteins in cultured insect cells (for example Sf9 cells) comprise the pAc series (Smith et al. (1983) Mol. Cell Biol.. 3:2156-2165) and the pVL series (Lucklow and Summers (1989) Virology 170:31-39).

The abovementioned vectors offer only a small overview over suitable vectors which are possible. Further plasmids are known to the skilled worker and are described, for example, in: Cloning Vectors (ed. Pouwels, P.H., et al., Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018). Further suitable expression systems for prokaryotic and eukaryotic cells, see in the chapters 16 and 17 of Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In a further embodiment of the process, the desaturases and/or elongases can be expressed in single-cell plant cells (such as algae), see Falciatore et al., 1999, Marine Biotechnology 1 (3):239-251 and references cited therein, and plant cells from higher plants (for example spermatophytes such as crops). Examples of plant expression vectors comprise those which are described in detail in: Becker, D., Kemper, E., Schell, J., and Masterson, R. (1992) "New plant binary vectors with selectable markers located proximal to the left border", Plant Mol. Biol. 20:1195-1197; and Bevan, M.W. (1984) "Binary Agrobacterium vectors for plant transformation", Nucl. Acids Res. 12:8711-8721; Vectors for Gene Transfer in Higher Plants; in: Transgenic Plants, vol. 1, Engineering and Utilization, ed.: Kung and R. Wu, Academic Press, 1993, pp. 15-38.

A plant expression cassette preferably comprises regulatory sequences which are capable of governing the gene expression in plant cells and which are linked operably so that each sequence can fulfill its function, such as transcriptional termination, for example polyadenylation signals. Preferred polyadenylation signals are those which originate from Agrobacterium tumefaciens T-DNA, such as the gene 3 of the Ti plasmid pTiACH5, which is known as octopine synthase (Gielen et al., EMBO J. 3 (1984) 835ff.) or functional equivalents thereof, but all other terminators which are functionally active in plants are also suitable.

Since plant gene expression is very often not limited to the transcriptional levels, a plant expression cassette preferably comprises other operably linked sequences such as translation enhancers, for example the overdrive sequence which comprises the 5'-untranslated leader sequence from tobacco mosaic virus, which increases the protein/RNA ratio (Gallie et al., 1987, Nucl. Acids Research 15:8693-8711).

As described above, plant gene expression must be linked operably with a suitable promoter which performs gene expression with the correct timing or in a cell- or tissue-specific manner. Utilizable promoters are constitutive promoters (Benfey et al., EMBO J. 8 (1989) 2195-2202) such as those which are derived from plant viruses, such as 35S CAMV (Franck et al., Cell 21 (1980) 285-294), 19S CaMV (see also US 5352605 and WO 84/02913) or plant promoters such as the Rubisco small subunit, which is described in US 4,962,028.

Other sequences which are preferred for the use for operable linkage in plant gene expression cassettes are targeting sequences, which are required for targeting the gene product into its relevant cell compartment (for a review see Kermode, Crit. Rev. Plant Sci. 15, 4 (1996) 285-423 and references cited therein), for example into the vacuole, the nucleus, all types of plastids, such as amyloplasts, chloroplasts, chromoplasts, the extracellular space, the mitochondria, the endoplasmic reticulum, oil bodies, peroxisomes and other plant cell compartments.

Plant gene expression can also be facilitated as described above via a chemically inducible promoter (for a review see Gatz 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48:89-108). Chemically inducible promoters are suitable in particular when it is desired that gene expression is clock-specific. Examples of such promoters are a salicylic acid-inducible promoter (WO

95/19443), a tetracyclin-inducible promoter (Gatz et al. (1992) Plant J. 2, 397-404) and an ethanol-inducible promoter.

Other promoters which are suitable are promoters which respond to biotic or abiotic stress conditions, for example the pathogen-induced PRP1 gene promoter (Ward et al., Plant. Mol. Biol. 22 (1993) 361-366), the heat-inducible tomato hsp80 promoter (US 5,187,267), the chill-inducible potato alpha-amylase promoter (WO 96/12814) or the wound-inducible pinII promoter (EP-A-0 375 091).

Preferred promoters are in particular those which bring about the expression of genes in tissues and organs in which lipid and oil biosynthesis takes place, in seed cells, such as cells of the endosperm and of the developing embryo. Suitable promoters are the oilseed rape napin gene promoter (US 5,608,152), the Vicia faba USP promoter (Baeumlein et al., Mol Gen Genet, 1991, 225 (3):459-67), the Arabidopsis oleosin promoter (WO 98/45461), the Phaseolus vulgaris phaseolin promoter (US 5,504,200), the Brassica Bce4 promoter (WO 91/13980) or the legumin B4 promoter (LeB4; Baeumlein et al., 1992, Plant Journal, 2 (2):233-9), and promoters which bring about the seed-specific expression in monocots such as maize, barley, wheat, rye, rice and the like. Suitable promoters which should be taken into consideration are the barley lpt2 or lpt1 gene promoter (WO 95/15389 and WO 95/23230), or those described in WO 99/16890 (promoters from the barley hordein gene, the rice glutelin gene, the rice oryzin gene, the rice prolamin gene, the wheat gliadin gene, the wheat glutelin gene, the maize zein gene, the oat glutelin gene, the sorghum kasirin gene, the rye secalin gene).

In particular, it may be desired to bring about the multiparallel expression of the desaturases and/or elongases used in the method alone or in combination with other desaturases or elongases. Such expression cassettes can be introduced via the simultaneous transformation of a plurality of individual expression constructs or, preferably, by combining a plurality of expression cassettes on one construct. Also, it is possible to transform a plurality of vectors with in each case a plurality of expression cassettes and to transfer them to the host cell.

Promoters which are likewise especially suitable are those which bring about the plastid-specific expression since plastids are the compartment in which the precursors and some end products of lipid biosynthesis are synthetized. Suitable promoters such as the viral RNA polymerase promoter are described in WO 95/16783

and WO 97/06250, and the Arabidopsis clpP promoter, described in WO 99/46394.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. The terms "transformation" and "transfection", conjugation and transduction, as used in the present context, are meant to comprise a multiplicity of methods known in the art for introducing foreign nucleic acid (for example DNA) into a host cell, including calcium phosphate or calcium chloride coprecipitation, DEAE-dextran-mediated transfection, lipofection, natural competence, chemically mediated transfer, electroporation or particle bombardment. Suitable methods for the transformation or transfection of host cells, including plant cells, can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual., 2nd edition., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) and other laboratory handbooks such as Methods in Molecular Biology, 1995, vol. 44, Agrobacterium protocols, ed.: Gartland and Davey, Humana Press, Totowa, New Jersey.

Host cells which are suitable in principle for taking up the nucleic acid according to the invention, the gene product according to the invention or the vector according to the invention are all prokaryotic or eukaryotic organisms. The host organisms which are advantageously used are organisms such as bacteria, fungi, yeasts or plant cells, preferably plants or parts thereof. Fungi, yeasts or plants are used by preference; especially preferably plants, very especially preferably plants such as oil crops which comprise large amounts of lipid compounds, such as oilseed rape, evening primrose, hemp, thistle, peanut, canola, linseed, soya, safflower, sunflower, borage or plants such as maize, wheat, rye, oats, triticale, rice, barley, cotton, cassava, pepper, Tagetes, Solanaceae plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa, bushy plants (coffee, cocoa, tea), Salix species, trees (oil palm, coconut) and perennial grasses and fodder crops. Especially preferred plants according to the invention are oil crops such as soya, peanut, oilseed rape, canola, linseed, hemp, evening primrose, sunflower, safflower, trees (oil palm, coconut).

Nucleic acid sequences which are advantageously used in the process according to the invention are those which encode polypeptides with a Δ6-desaturase activity, Δ6-elongase activity or Δ5-desaturase activity, selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29 or SEQ ID NO: 31,

b) nucleic acid sequences which, owing to the degeneracy of the genetic code, are obtained by back translation of the amino acid sequences shown in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32,

c) derivatives of the nucleic acid sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29 or SEQ ID NO: 31 which encode polypeptides with the amino acid sequences shown in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32 and which have at least 50% homology at the amino acid level, without the enzymatic activity of the polypeptides being substantially reduced.

The abovementioned nucleic acid according to the invention originates from organisms such as animals, ciliates, fungi, plants such as algae or dinoflagellates which are capable of synthesizing PUFAs.

The term "nucleic acid (molecule)" as used in the present context also comprises the untranslated sequence located at the 3' and at the 5' end of the coding gene region: at least 500, preferably 200, especially preferably 100 nucleotides of the sequence upstream of the 5' terminus of the coding region and at least 100, preferably 50, especially preferably 20 nucleotides of the sequence downstream of the 3' end of the coding gene region. An "isolated" nucleic acid molecule is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. An "isolated" nucleic acid preferably has no sequences which naturally flank the nucleic acid in the genomic DNA of the organism from which the nucleic acid originates (for example sequences which are present at the 5' and 3' ends of the nucleic acid). In different embodiments, the isolated desaturase

or elongase nucleic acid molecule may comprise, for example less than approximately 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in the genomic DNA of the cell from which the nucleic acid originates.

The nucleic acid molecules used in the process, for example a nucleic acid molecule with a nucleotide sequence of the SEQ ID NO: 1 or a part thereof, can be isolated using molecular-biological standard techniques and the sequence information provided herein. Also, for example a homologous sequence or homologous, conserved sequence regions at the DNA or amino acid level can be identified with the aid of comparative algorithms. They can be used as hybridization probe and standard hybridization techniques (as described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) for the isolation of further nucleic acid sequences which are useful in the process. Moreover, a nucleic acid molecule comprising a complete sequence of the SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 or a part thereof can be isolated by polymerase chain reaction, where oligonucleotide primers, which are used on the basis of this sequence or parts thereof (for example, it is possible to isolate a nucleic acid molecule comprising the complete sequence or a part thereof by means of polymerase chain reaction using oligonucleotide primers which have been generated on the basis of the same sequence). For example, mRNA can be isolated from cells (for example by means of the guanidinium thiocyanate extraction method of Chirgwin et al. (1979) Biochemistry 18:5294-5299) and cDNA can be generated by means of reverse transcriptase (for example Moloney MLV Reverse Transcriptase, available from Gibco/BRL, Bethesda, MD, or AMV Reverse Transcriptase, available from Seikagaku America, Inc., St.Petersburg, FL). Synthetic oligonucleotide primers for amplification by means of polymerase chain reaction can be generated based on one of the sequences shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 and that of Figure 5a, or with the aid of the amino acid sequences shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32. A nucleic acid according to the invention can be amplified in accordance with standard PCR amplification techniques using cDNA or, alternatively, genomic DNA as template and suitable oligonucleotide primers. The nucleic acid amplified thus can be cloned into a suitable vector and characterized by means of DNA sequence analysis. Oligonucleotides which correspond to a desaturase nucleotide sequence can be generated by means of

synthetic standard methods, for example using an automatic DNA synthesizer.

Homologs of the desaturase or elongase nucleic acid sequences used, with sequence SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31, means for example allelic variants with at least approximately 50 to 60%, preferably at least approximately 60 to 70%, more preferably at least approximately 70 to 80%, 80 to 90% or 90 to 95% and even more preferably at least approximately 95%, 96%, 97%, 98%, 99% or more homology with one of the nucleotide sequences shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 or their homologs, derivatives or analogs, or parts of these. Moreover, isolated nucleic acid molecules of a nucleotide sequence which hybridize with one of the nucleotide sequences shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 or part thereof, hybridize for example under stringent conditions. Allelic variants comprise in particular functional variants which can be obtained by deletion, insertion or substitution of nucleotides from/into the sequence shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31, it being intended, however, that the enzyme activity of the resulting synthesized proteins is advantageously retained for the insertion of one or more genes. Proteins which retain the enzymatic activity of the desaturase or elongase, i.e. whose activity is essentially not reduced, means proteins with at least 10%, preferably 20%, especially preferably 30%, very especially preferably 40% of the original enzyme activity in comparison with the protein encoded by SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32.

Homologs of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 mean for example also bacterial, fungal and plant homologs, truncated sequences, single-stranded DNA or RNA of the coding and noncoding DNA sequence.

Homologs of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 also means derivatives such as, for example, promoter variants. The promoters upstream of the abovementioned nucleotide sequences can be modified by one or more nucleotide substitutions, insertion(s) and/or deletion(s) without, however, interfering with the functionality or activity of the promoters. Moreover, it is possible to increase the activity of the promoters by modifying their sequence or to replace them completely by more active promoters, including promoters from heterologous organisms.

The abovementioned nucleic acids and protein molecules with desaturase or elongase activity which are involved in the metabolism of lipids and fatty acids, PUFA cofactors and enzymes or in the transport of lipophilic compounds across membranes are used in the process according to the invention for the modulation of the production of compounds of the general formula I in transgenic plants such as maize, wheat, rye, oats, triticale, rice, barley, soybean, peanut, cotton, Linum species such as linseed or flax, Brassica species such as oilseed rape, canola and turnip rape, pepper, sunflower, borage, evening primrose and Tagetes, Solanaceae plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, cassava, alfalfa, bushy plants (coffee, cocoa, tea), Salix species, trees (oil palm, coconut) and perennial grasses and fodder crops, either directly (for example when the overexpression or optimization of a fatty acid biosynthesis protein has a direct effect on the yield, production and/or production efficiency of the fatty acid from modified organisms) and/or can have an indirect effect which nevertheless leads to an increase in the yield, production and/or production efficiency of a desired compound or a decrease in undesired compounds (for example when the modulation of the metabolism of lipids and fatty acids, cofactors and enzymes leads to modifications of the yield, production and/or production efficiency or the composition of the desired compounds within the cells, which, in turn, may have an effect on the production of one or more fatty acids).

The combination of different precursor molecules and biosynthetic enzymes results in the production of different fatty acid molecules, which has a decisive effect on lipid composition. Since polyunsaturated fatty acids (= PUFAs) are not simply incorporated into triacylglycerol, but also into membrane lipids.

Lipid synthesis can be divided into two sections: the synthesis of fatty acids and their binding to sn-glycerol-3-phosphate, and the addition or modification of a polar head group. Conventional lipids which are used in membranes comprise phospholipids, glycolipids, sphingolipids and phosphoglycerides. Fatty acid synthesis starts with the conversion of acetyl-CoA into malonyl-CoA by the enzyme acetyl-CoA carboxylase or into acetyl-ACP by the enzyme acetyl transacylase. After a condensation reaction, these two product molecules together form acetoacetyl-ACP, which is converted by a series of condensation, reduction and dehydratization reactions so that a saturated fatty acid molecule with the desired chain length is obtained. The production of the unsaturated fatty acids from these molecules is catalyzed by specific desaturases, either aerobically by means of

molecular oxygen or anaerobically (as regards the fatty acid synthesis in microorganisms, see F.C. Neidhardt et al. (1996) E. coli and Salmonella. ASM Press: Washington, D.C., pp. 612-636 and references cited therein; Lengeler et al. (ed.) (1999) Biology of Procaryotes. Thieme: Stuttgart, New York, and references therein, and Magnuson, K., et al. (1993) Microbiological Reviews 57:522-542 and the references therein).

Examples of precursors for PUFA biosynthesis are oleic acid, linoleic acid and linolenic acid. These C_{18}-carbon fatty acids must be elongated to C_{20} and C_{22} to obtain fatty acids of the eicosa and docosa chain type. With the aid of the desaturases used in the process, such as Δ5- and Δ6-desaturase and Δ6-elongase, it is possible to obtain arachidonic acid and eicosapentaenoic acid and various other long-chain PUFAs, to extract them and to use them for various purposes in applications in foodstuffs, feeding stuffs, cosmetics or pharmacology. Using the abovementioned enzymes, it is possible to produce preferably C_{18} + C_{20} fatty acids with at least two, three, four or five double bonds in the fatty acid molecule, preferably C_{20}-fatty acids with advantageously three, four or five double bonds in the fatty acid molecule. Desaturation can take place before or after elongation of the fatty acid in question. This is why the products of desaturase activities and the further desaturation and elongation which are possible give rise to preferred PUFAs with a higher degree of desaturation, including a further elongation from C_{20} to C_{22}-fatty acids, to give fatty acids such as γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, stearidonic acid, eicosatetraenoic acid or eicosapentaenoic acid. Substrates in the process according to the invention are, for example, linoleic acid, γ-linolenic acid, α-linolenic acid, dihomo-γ-linolenic acid, eicosatetraenoic acid or stearidonic acid. Preferred substrates are linoleic acid, γ-linolenic acid and/or α-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid, eicosatetraenoic acid or eicosapentaenoic acid, respectively. The C_{18}- or C_{20}-fatty acids with at least two double bonds in the fatty acid are obtained in the process according to the invention in the form of the free fatty acid or in the form of its esters (see formula I), for example in the form of its glycerides.

The term "glyceride" is understood as meaning a glycerol which is esterified with one, two or three carboxylic acid residues (mono-, di- or triglyceride). "Glyceride" is also understood as being a mixture of various glycerides. The glyceride, or glyceride mixture, may comprise further additions, for example

free fatty acids, antioxidants, proteins, carbohydrates, vitamins and/or other substances.

A "glyceride" for the purposes of the process according to the invention is furthermore understood as meaning glycerol-derived derivatives. These include, in addition to the above-described fatty acid glycerides, glycerophospholipids and glyceroglycolipids. Preferred examples which may be mentioned in this context are the glycerophospholipids such as lecithin (phosphatidylcholine), cardiolipin, phosphatidylglycerol, phosphatidylserine and alkylacylglycerophospholipids.

Furthermore, fatty acids must subsequently be translocated to various sites of modification and incorporated into the triacylglycerol storage lipid. A further important step in lipid synthesis is the transfer of fatty acids on the polar head groups, for example by the enzyme glycerol fatty acid acyltransferase (see Frentzen, 1998, Lipid, 100(4-5):161-166).

Publications on plant fatty acid biosynthesis, desaturation, the lipid metabolism and membrane transport of lipidic compounds, beta-oxidation, fatty acid modification and cofactors, triacylglycerol storage and triacylglycerol assembly including the references cited therein, see the following papers: Kinney, 1997, Genetic Engeneering, ed.: JK Setlow, 19:149-166; Ohlrogge and Browse, 1995, Plant Cell 7:957-970; Shanklin and Cahoon, 1998, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49:611-641; Voelker, 1996, Genetic Engeneering, ed.: JK Setlow, 18:111-13; Gerhardt, 1992, Prog. Lipid R. 31:397-417; Gühnemann-Schäfer & Kindl, 1995, Biochim. Biophys Acta 1256:181-186; Kunau et al., 1995, Prog. Lipid Res. 34:267-342; Stymne et al., 1993, in: Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants, ed.: Murata and Somerville, Rockville, American Society of Plant Physiologists, 150-158, Murphy & Ross 1998, Plant Journal. 13(1):1-16.

The PUFAs produced in the process comprise a group of molecules which higher animals are no longer capable of synthesizing and must therefore take up, or which higher animals can no longer synthesize themselves in sufficient amounts and must thus additionally take them up, although they are synthesized readily by other organisms such as bacteria; for example, cats are no longer capable of synthesizing arachidonic acid.

For the purposes of the invention, the terms "desaturase or elongase" or "desaturase or elongase polypeptide" comprises proteins which are implicated in the desaturation and elongation

of fatty acids, and their homologs, derivatives or analogs. The terms desaturase or elongase nucleic acid sequence(s) comprise nucleic acid sequences which encode a desaturase or elongase and in which a part can be a coding region and likewise corresponding 5'- and 3'-untranslated sequence regions. The terms production or producitivity are known in the art and comprise the concentration of the fermentation product (compound of the formula I) which is formed within a specified period of time and a specified fermentation volume (for example kg of product per hour per liter). The term production efficiency comprises the time span required for obtaining a specific amount of product (for example the time required by the cell for establishing a certain throughput rate of a fine chemical). The term yield or product/carbon yield is known in the art and comprises the efficiency with which the carbon source is converted into the product (i.e. the fine chemical). This is usually expressed as, for example, kg of product per kg of carbon source. Increasing the yield or production of the compound results in increasing the amount of resulting molecules or the suitable resulting molecules of this compound in a certain amount of culture over a specified period. The terms biosynthesis or biosynthetic pathway are known in the art and comprise the synthesis of a compound, preferably an organic compound, by a cell starting from intermediates, for example in a multi-step process which is strongly regulated. The terms catabolism or catabolic pathway are known in the art and comprise the cleavage of a compound, preferably an organic compound, by a cell to give catabolites (in more general germs, smaller or less complex molecules), for example in a multi-step process which is strongly regulated. The term metabolism is known in the art and comprises the totality of the biochemical reactions which take place in an organism. The metabolism of a certain compound (for example the metabolism of a fatty acid) thus comprises the totality of the biosynthetic pathways, modified pathways and catabolic pathways of this compound in the cell which relate to this compound.

In a further embodiment, derivatives of the nucleic acid molecule according to the invention encode proteins with at least 50%, advantageously approximately 50 to 60%, preferably at least approximately 60 to 70% and more preferably at least approximately 70 to 80%, 80 to 90%, 90 to 95% and most preferably at least approximately 96%, 97%, 98%, 99% or more homology (= identity) with a complete amino acid sequence of the SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32. The homology of the amino acid sequence can be determined over the entire sequence region using the program PileUp (J. Mol. Evolution., 25, 351-360, 1987, Higgins et al., CABIOS, 5,

1989:151–153) or BESTFIT or GAP (Henikoff, S. and Henikoff, J. G. (1992). Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. USA 89: 10915-10919.)

Moreover, the invention comprises nucleic acid molecules which differ from one of the nucleotide sequences shown in SEQ ID NO: 1, 3, 5 or 11 (and parts thereof) as the result of the degeneracy of the genetic code and which thus encode the same desaturase as the desaturase which is encoded by the nucleotide sequences shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31.

In addition to the desaturase nucleotide sequences shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31, the skilled worker will recognize that DNA sequence polymorphisms which result in modifications in the amino acid sequences of the desaturases or elongases may exist within a population. These genetic polymorphisms in the desaturase or elongase gene may exist between individuals within a population as the result of natural variation. These natural variants usually bring about a variance of from 1 to 5% in the nucleotide sequence of the desaturase or elongase gene. All and sundry of these nucleotide variations and resulting amino acid polymorphisms in the enzyme desaturase or elongase which are the result of natural variation and which do not modify the functional activity of desaturases or elongases are also intended to fall under the scope of the invention.

Nucleic acid molecules which are advantageous for the process according to the invention can be isolated on the basis of their homology with the desaturase or elongase nucleic acids disclosed herein using the sequences or part thereof as hybridization probe, following standard hybridization techniques under stringent hybridization conditions. In this context, it is possible for example to use isolated nucleic acid molecules which are at least 15 nucleotides in length and which hybridize under stringent conditions with the nucleic acid molecules which comprise a nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31. It is also possible to use nucleic acids with at least 25, 50, 100, 250 or more nucleotides. The term "hybridizes under stringent conditions" as used in the present context is understood as describing hybridization and wash conditions under which nucleotide sequences with at least 60% homology with one another usually remain hybridized with one another. The conditions are preferably such that sequences which are at least approximately 65%, more preferably at least approximably 70% and even more preferably at

least approximately 75% or more homologous with one another usually remain hybridized with one another. These stringent conditions are known to the skilled worker and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N. Y. (1989), 6.3.1-6.3.6. A preferred nonlimiting example of stringent hybridization conditions is hybridization in 6 x sodium chloride/sodium citrate (SSC) at approximately 45°C, followed by one or more wash steps in 0.2 x SSC, 0.1% SDS at 50 to 65°C. The skilled worker knows that these hybridization conditions differ depending on the type of the nucleic acid and, for example when organic solvents are used, with regard to the temperature and concentration of the buffer. For example, under "standard hybridization conditions" the temperature differs depending on the type of nucleic acid between 42°C and 58°C in aqueous buffer with a concentration of 0.1 to 5 x SSC (pH 7.2). If organic solvent is present in the abovementioned buffer, for example 50% formamide, the temperature under standard conditions is approximately 42°C. The hybridization conditions for DNA:DNA hybrids preferably are for example 0.1 x SSC and 20°C to 45°C, preferably between 30°C and 45°C. The hybridization conditions for DNA:RNA hybrids preferably are for example 0.1 x SSC and 30°C to 55°C, preferably between 45°C and 55°C. The abovementioned hybridization temperatures are determined for example for a nucleic acid with a length of approximately 100 bp (= base pairs) and a G + C content of 50% in the absence of formamide. The skilled worker knows how to identify the hybridization conditions required with the aid of textbooks, such as the one mentioned above, or the following textbooks: Sambrook et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1989; Hames and Higgins (ed.) 1985, "Nucleic Acids Hybridization: A Practical Approach", IRL Press at Oxford University Press, Oxford; Brown (ed.) 1991, "Essential Molecular Biology: A Practical Approach", IRL Press at Oxford University Press, Oxford.

To determine the percentage homology (= identity) of two amino acid sequences (for example of the sequences of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32) or of two nucleic acids (for example one of the sequences of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31), the sequences are written underneath each other to provide an optimal comparison (for example, gaps may be introduced into the sequence of a protein or a nucleic acid in order to generate an optimal alignment with the other protein or the other nucleic acid). The amino residues of nucleotides at the corresponding amino acid positions or nucleotide positions are then compared. If a position in a sequence is occupied by the same amino acid residue or the same nucleotide as the corresponding position in the other

sequence, the molecules are homologous at this position (i.e. amino acid or nucleic acid "homology" as used in the present context corresponds to amino acid or nucleic acid "identity"). The percentage homology between the two sequences is a function of the number of identical positions which the sequences share (i.e. percent homology = number of identical positions/total number of positions x 100). The terms homology and identity are thus to be regarded as synonymous.

An isolated nucleic acid molecule which encodes a desaturase or elongase which is homologous to a protein sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32 can be generated by introducing one or more nucleotide substitutions, additions or deletions into a nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 so that one or more amino acid substitutions, additions or deletions are introduced into the protein which is encoded. Mutations can be introduced into one of the sequences of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 by standard techniques such as site-specific mutagenesis and PCR-mediated mutagenesis. It is preferred to generate conservative amino acid substitutions at one or more of the predicted nonessential amino acid residues. In a "conservative amino acid substitution", the amino acid residue is substituted by an amino acid residue with a similar side chain. Families of amino acid residues with similar side chains have been defined in the art. These families comprise amino acids with basic side chains (for example lysine, arginine, histidine), acidic side chains (for example aspartic acid, glutamic acid), uncharged polar side chains (for example glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), unpolar side chains (for example alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (for example threonine, valine, isoleucine) and aromatic side chains (for example tyrosine, phenylalanine, tryptophan, histidine). A predicted nonessential amino acid residue in a desaturase or elongase is thus preferably substituted by another amino acid residue from the same family of side chains. As an alternative, the mutations can, in a different embodiment, be introduced randomly over the entire desaturase-encoding sequence or part thereof, for example by means of saturation mutagenesis, and the resulting mutants can be screened for the desaturase activity described herein in order to identify mutants which retain the desaturase or elongase activity. After the mutagenesis of one of the sequences of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29 or 31 the encoded protein can be

expressed recombinantly, and the activity of the protein can be determined for example using the assays described herein.

The invention is illustrated further by the examples which follow, but which are not to be construed as limiting. The content of all of the references, patent applications, patents and published patent applications cited in the present patent application is herein incorporated by reference.

Examples section

Example 1: General methods

a) General cloning methods:

Cloning methods such as, for example, restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids onto nitrocellulose and nylon membranes, linking of DNA fragments, transformation of Escherichia coli and yeast cells, bacterial cultures and sequence analysis of recombinant DNA were carried out as described in Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) or Kaiser, Michaelis and Mitchell (1994) "Methods in Yeast Genetics" (Cold Spring Harbor Laboratory Press: ISBN 0-87969-451-3).

b) Chemicals

Unless otherwise stated in the text, the chemicals used were obtained in analytical-grade quality from Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen). Solutions were made with purified, pyrogen-free water, hereinbelow referred to as H_2O, from a Milli-Q Water System water purification system (Millipore, Eschborn). Restriction endonucleases, DNA-modifying enzymes and molecular biology kits were obtained from AGS (Heidelberg), Amersham (Braunschweig), Biometra (Göttingen), Boehringer (Mannheim), Genomed (Bad Oeynhausen), New England Biolabs (Schwalbach/Taunus), Novagen (Madison, Wisconsin, USA), Perkin-Elmer (Weiterstadt), Pharmacia (Freiburg), Qiagen (Hilden) and Stratagene (Amsterdam, Netherlands). Unless otherwise specified, they were used in accordance with the manufacturer's instructions.

Example 2: Isolation of total RNA and poly(A)$^+$-RNA from plants

Total RNA is isolated from plants such as linseed and oilseed rape and the like following a method described by Logemann et al. (1987, Anal. Biochem. 163, 21). The total RNA can be obtained from protonemal tissue from moss using the GTC method (Reski et al., 1994, Mol. Gen. Genet., 244:352-359).

Example 3: Transformation of Agrobacterium

The Agrobacterium-mediated transformation of plants can be carried out for example using the Agrobacterium tumefaciens strain GV3101- (pMP90-) (Koncz and Schell, Mol. Gen. Genet. 204 (1986) 383-396) or LBA4404- (Clontech) or C58C1 pGV2260 (Deblaere et al 1984, Nucl. Acids Res. 13, 4777-4788)). The transformation can be carried out by standard transformation techniques (also Deblaere et al. 1984).

Example 4: Plant transformation

The Agrobacterium-mediated transformation of plants can be carried out using standard transformation and regeneration techniques (Gelvin, Stanton B., Schilperoort, Robert A., Plant Molecular Biology Manual, 2nd ed., Dordrecht: Kluwer Academic Publ., 1995, in Sect., Ringbuch Zentrale Signatur: BT11-P ISBN 0-7923-2731-4; Glick, Bernard R., Thompson, John E., Methods in Plant Molecular Biology and Biotechnology, Boca Raton: CRC Press, 1993, 360 S., ISBN 0-8493-5164-2).

Oilseed rape can be transformed by means of cotyledon or hypocotyl transformation (Moloney et al., Plant Cell 8 (1989) 238-242; De Block et al., Plant Physiol. 91 (1989) 694-701). The use of antibiotics for the seletion of agrobacteria and plants depends on the Agrobacterium strain and the binary vector used for the transformation. Normally, oilseed rape is selected using kanamycin as selectable plant marker.

The Agrobacterium-mediated gene transfer into linseed (Linum usitatissimum) can be carried out using for example a technique described by Mlynarova et al. (1994) Plant Cell Report 13:282-285.

The transformation of soya can be carried out using for example a technique described in EP-A-0 0424 047 (Pioneer Hi-Bred International) or in EP-A-0 0397 687, US 5,376,543, US 5,169,770 (University Toledo).

The transformation of plants using particle bombardment, polyethylene glycol mediated DNA uptake or via the silicon carbonate fiber technique is described for example by Freeling and Walbot "The maize handbook" (1993) ISBN 3-540-97826-7, Springer Verlag New York).

Example 5: Plasmids for plant transformation

Binary vectors such as pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990) 221-230) or pGPTV (Becker et al 1992, Plant Mol. Biol. 20:1195-1197) can be used for plant transformation. The binary vectors can be constructed by ligating the cDNA in sense or antisense orientation into T-DNA. 5' of the cDNA, a plant promoter activates cDNA transcription. A polyadenylation sequence is located 3' of the cDNA. The binary vectors can bear different marker genes. In particular, the nptII marker gene, which encodes kanamycin resistance conferred by neomycin phosphotransferase, can be substituted by the herbicide-resistant form of an acetolactate synthase gene (AHAS or ALS). The ALS gene is described in Ott et al., J. Mol. Biol. 1996, 263:359-360. The v-ATPase-c1 promoter can be cloned into plasmid pBin19 or pGPTV and used for the expression of the marker gene by cloning upstream of the ALS coding region. The abovementioned promoter corresponds to a 1153 base-pair fragment from Beta vulgaris (Plant Mol Biol, 1999, 39:463-475). In this context, not only sulfonylureas, but also imidazolinones such as imazethapyr or sulphonylureas may be used as antimetabolites for the selection.

Tissue-specific expression can be achieved using a tissue-specific promoter. For example, seed-specific expression can be achieved by cloning the DC3 or LeB4 or USP promoter or the phaseolin promoter 5' of the cDNA. However, any other seed-specific promoter element such as, for example, the napin or arcelin promoter (Goossens et al. 1999, Plant Phys. 120(4):1095-1103 and Gerhardt et al. 2000, Biochimica et Biophysica Acta 1490(1-2):87-98) may also be used. The CaMV-35S promoter or a v-ATPase C1 promoter can be used for constitutive expression in the intact plants.

In particular, genes encoding desaturases and elongases can be cloned into a binary vector one after the other by constructing a plurality of expression cassettes in order to mimic the metabolic pathway in plants.

Within an expression casette, the protein to be expressed can be targeted into a cellular compartment using a signal peptide, for example for plastids, mitochondria or the endoplasmic reticulum

(Kermode, Crit. Rev. Plant Sci. 15, 4 (1996) 285-423). The signal peptide is cloned 5' in the reading frame with the cDNA to achieve the subcellular localization of the fusion protein.

Examples of multiexpression cassettes are given hereinbelow.

I.) Promoter-terminator cassettes

Expression cassettes consist of least two functional units such as a promoter and a terminator. Further desired gene sequences such as targeting sequences, coding regions of genes or parts thereof and the like can be inserted between promoter and terminator. To construct expression cassettes, promoters and terminators (USP promoter: Baeumlein et al., Mol Gen Genet, 1991, 225 (3):459-67); OCS terminator: Gielen et al. EMBO J. 3 (1984) 835ff.) are isolated with the aid of the polymerase chain reaction and tailor-made with flanking sequences of choice on the basis of synthetic oligonucleotides.

Examples of oligonucleotides which can be used are the following:

USP1 upstream: CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA

USP2 upstream: CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA

USP3 upstream: CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA

USP1 downstream: AAAACTGCAGGCGGCCGCCCACCGCGGTGGGCTGGCTATGAAGAAATT

USP2 downstream: CGCGGATCCGCTGGCTATGAAGAAATT

USP3 downstream: TCCCCCGGGATCGATGCCGGCAGATCTGCTGGCTATGAAGAAATT

OCS1 upstream: AAAACTGCAGTCTAGAAGGCCTCCTGCTTTAATGAGATAT

OCS2 upstream:
CGCGGATCCGATATCGGGCCCGCTAGCGTTAACCCTGCTTTAATGAGATAT

OCS3 upstream: TCCCCCGGGCCATGGCCTGCTTTAATGAGATAT

OCS1 downstream:
CCCAAGCTTGGCGCGCCGAGCTCGAATTCGTCGACGGACAATCAGTAAATTGA

OCS2 downstream:
CCCAAGCTTGGCGCGCCGAGCTCGAATTCGTCGACGGACAATCAGTAAATTGA

OCS3 downstream: CCCAAGCTTGGCGCGCCGAGCTCGTCGACGGACAATCAGTAAATTGA

The methods are known to the specialist worker and are generally known from the literature.

In a first step, a promoter and a terminator are amplified via PCR. Then, the terminator is cloned into a recipient plasmid and, in a second step, the promoter is inserted upstream of the terminator. This gives an expression cassette on a plasmid vehicle. The plasmids pUT1, 2 and 3 are generated on the basis of the plasmid pUC19.

The constructs are defined in accordance with the invention in SEQ ID NO: 33, 34 and 42. They comprise the USP promoter and the OCS terminator. Based on these plasmids, the construct pUT12 is generated by cutting pUT1 with SalI/ScaI and cutting pUT2 with XhoI/ScaI. The fragments in the expression cassettes are ligated and transformed into E. coli XLI blue MRF. After picking out ampicillin-resistant colonies, DNA is prepared, and those clones which comprise two expression cassettes are identified by restriction analysis. The XhoI/SalI ligation of compatible ends has eliminated the two cleavage sites XhoI and SalI between the expression cassettes. This gives rise to plasmid pUT12, which is defined in SEQ ID NO: 36. pUT12 is subsequently cut again with SalI/ScaI and pUT3 with XhoI/ScaI. The fragments comprising the expression cassettes are ligated and transformed into E. coli XLI blue MRF. After singling out ampicillin-resistant colonies, DNA is prepared, and those clones which comprise three expression cassettes are identified by restriction analysis. In this manner, a set of multiexpression cassettes is created which can be exploited for inserting the desired DNA and is described in Table 1 and can additionally incorporate further expression cassettes.

They comprise the following elements:

Table 1

pUC19 derivate	Cleavage sites before the USP promoter	Multiple cloning cleavage sites	Cleavage sites behind the OCS terminator
pUT1	EcoRI/AscI/ SacI/XhoI	BstXI/NotI/ PstI/XbaI/StuI	SalI/EcoRI/ SacI/AscI/ HindIII
pUT2	EcoRI/AscI/ SacI/XhoI	BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/ SacI/AscI/ HindIII
pUT3	EcoRI/AscI/ SacI/XhoI	BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/ AscI/HindIII
pUT12 Double expression cassette	EcoRI/AscI/ SacI/XhoI	BstXI/NotI/ PstI/XbaI/StuI and BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/ SacI/AscI/ HindIII
pUT123 Triple expression cassette	EcoRI/AscI/ SacI/XhoI	1.BstXI/NotI/ PstI/XbaI/StuI and 2.BamHI/EcoRV/ ApaI/NheI/ HpaI and 3.BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII

Furthermore, further multiexpression cassettes can be generated and employed for seed-specific gene expression, as described and as specified in greater detail in Table 2, with the aid of the

i) USP promoter or with the aid of the
ii) 700 base pair 3' fragment of the LeB4 promoter or with the aid of the
iii) DC3 promoter.

The DC3 promoter is described in Thomas, Plant Cell 1996, 263:359-368 and consists merely of the region -117 to +26, which is why it therefore constitutes one of the smallest known seed-specific promoters. The expression cassettes can comprise several copies of the same promoter or else be constructed via three different promoters.

The vectors used for the transformation of plants and the sequences of the inserted genes/proteins can be found in sequence listing SEQ ID NO: 43 to 49.

Advantageously used polylinker or polylinker-terminator-polylinkers can be found in the sequences SEQ ID NO: 50 to 52.

Table 2: Multiple expression cassettes

Plasmid name of the pUC19 derivative	Cleavage sites before the respective promoter	Multiple cloning cleavage sites	Cleavage sites behind the OCS terminator
pUT1 (pUC19 with USP-OCS1)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/PstI/ XbaI/StuI	SalI/EcoRI/SacI/AscI/ HindIII
pDCT (pUC19 with DC3-OCS)	EcoRI/AscI/SacI/XhoI	(2) BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/SacI/AscI/ HindIII
pLeBT (pUC19-with LeB4(700)-OCS)	EcoRI/AscI/SacI/XhoI	(3) BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII
pUD12 (pUC 19 with USP-OCS1 and with DC3-OCS)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/ PstI/XbaI/StuI and (2) BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/SacI/AscI/ HindIII
pUDL123 Triple expression cassette (pUC19 with USP/DC3 and LeB4-700)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/ PstI/XbaI/StuI and (2) BamHI/ (EcoRV*)/ApaI/ NheI/HpaI and (3) BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII

* EcoRV cleavage site in the 700 base-pair fragment of the LeB4 promoter (LeB4-700)

Further promoters for multi-gene constructs can be generated analogously, in particular using the

a) 2.7 kb fragment of the LeB4 promoter or with the aid of the
b) phaseolin promoter or with the aid of the
c) constitutive v-ATPase c1 promoter.

It may be particularly desirable to use further especially suitable promoters for constructing seed-specific multi-expression cassettes such as, for example, the napin promoter or the arcelin-5 promoter.

II) Generation of expression constructs which comprise promoter, terminator and desired gene sequence for the expression of PUFA genes in plant expression cassettes.

In pUT123, the Δ6-elongase Pp_PSE1 is first inserted into the first cassette via BstXI and XbaI. Then, the moss Δ-6-desaturase (Pp_des6) is inserted into the second cassette via BamHI/NaeI,

and, finally, the Phaeodactylum Δ5-desaturase (Pt_des5) is inserted into the third cassette via BglII/NcoI. The triple construct is named pARA1. Taking into consideration sequence-specific restriction cleavage sites, further expression cassettes are shown in Table 3, which are named pARA2, pARA3 and pARA4, can be generated.

Table 3: Combinations of desaturases and elongases

Gene plasmid	Δ6-Desaturase	Δ5-Desaturase	Δ6-Elongase
pARA1	Pp_des6	Pt_des5	Pp_PSE1
pARA2	Pt_des6	Pt_des5	Pp_PSE1
pARA3	Pt_des6	Ce_des5	Pp_PSE1
pARA4	Ce_des6	Ce_des5	Ce_PSE1

Pp = Physcomitrella patens, Pt = Phaeodactylum tricornutum
Pp_PSE1 corresponds to the sequence of SEQ ID NO: 9.
PSE = PUFA-specific Δ6-elongase
Ce_des5 = Δ5-desaturase from Caenorhabditis elegans (Genbank Acc. No. AF078796)
Ce_des6 = Δ6-desaturase from Caenorhabditis elegans elegans (Genbank Acc. No. AF031477, bases 11-1342)
Ce_PSE1 = Δ6-elongase from Caenorhabditis elegans (Genbank Acc. No. AF244356, bases 1-867)

Further desaturases or elongase sequences can also be inserted into the expression cassettes in the described manner, such as, for example, Genbank Acc. Nr. AF231981, NM_013402, AF206662, AF268031, AF226273, AF110510 or AF110509.

iii) Transfer of expression cassettes into vectors for the transformation of Agrobacterium tumefaciens and for the transformation of plants

The constructs generated thus are inserted into the binary vector pGPTV by means of AscI. For this purpose, the multiple cloning sequence is extended by an AscI cleavage site. For this purpose, the polylinker is synthesized *de novo* as two double-stranded oligonucleotides, thereby introducing an additional AscI DNA sequence. The oligonucleotide is inserted into the vector pGPTV by means of EcoRI and HindIII. The cloning techniques required are known to the skilled worker and can simply be found in the literature as described in Example 1.

Example 6: Studying the expression of a recombinant gene product in a transformed organism

The activity of a recombinant gene product in the transformed host organism can be measured at the transcriptional and/or the translational level.

A suitable method for determining the extent to which the gene is transcribed (which indicates the amount of RNA which is available for the translation of the gene product) is to carry out a Northern blot as detailed hereinbelow (as reference, see Ausubel et al. (1988) Current Protocols in Molecular Biology, Wiley: New York, or the abovementioned Examples Section), where a primer which is designed in such a way that it binds to the gene of interest is labeled with a detectable label (usually a radioactive label or a chemiluminescent label) so that, when the total RNA of a culture of the organism is extracted, separated on a gel, transferred onto a stable matrix and incubated with this probe, the binding and extent of the binding of the probe indicates the existence and also the amount of the mRNA for this gene. This information indicates the degree to which the transformed gene has been transcribed. Cellular total RNA can be prepared from cells, tissues or organs using a plurality of methods, all of which are known in the art, such as, for example, the method of Bormann, E.R., et al. (1992) Mol. Microbiol. 6:317-326.

Northern hybridization:

To carry out the RNA hybridization, 20 µg of total RNA or 1 µg of poly(A)$^+$ RNA were separated by gel electrophoresis in agarose gels with a strength of 1.25% using formaldehyde, as described in Amasino (1986, Anal. Biochem. 152, 304), capillary-blotted onto positively charged nylon membranes Hybond N+, Amersham, Braunschweig) using 10 x SSC, immobilized using UV-light and prehybridized for 3 hours at 68°C using hybridization buffer (10% dextran sulfate w/v, 1 M NaCl, 1% SDS, 100 mg herring sperm DNA). The DNA probe was labeled with the Highprime DNA labeling kit (Roche, Mannheim, Germany) during the prehybridization step, using alpha-^{32}P-dCTP (Amersham, Braunschweig, Germany). After the labeled DNA probe had been added, the hybridization was carried out overnight at 68°C in the same buffer. The wash steps were carried out twice for 15 minutes using 2 x SSC and twice for 30 minutes using 1 x SSC, 1% SDS, at 68°C. The sealed filters were exposed at -70°C for a period of 1 to 14 days.

Standard techniques, such as a Western blot, can be employed for studying the presence or the relative amount of protein translated by this mRNA (see, for example, Ausubel et al. (1988) Current Protocols in Molecular Biology, Wiley: New York). In this method, the cellular total proteins are extracted, separated by gel electrophoresis, transferred to a matrix such as nitrocellulose and incubated with a probe such as an antibody which binds specifically to the desired protein. This probe is usually provided with a chemiluminescent or colorimetric label which can be detected readily. The presence and the amount of the label observed indicates the presence and the amount of the desired mutated protein present in the cell.

Example 7: Analysis of the effect of the recombinant proteins on the production of the desired product

The effect of the genetic modification in plants, fungi, algae, ciliates or on the production of a desired compound (such as a fatty acid) can be determined by growing the modified microorganisms or the modified plant under suitable conditions (such as those described above) and analyzing the medium and/or the cellular components for the increased production of the desired product (i.e. of lipids or a fatty acid). These analytical techniques are known to the skilled worker and comprise spectroscopy, thin-layer chromatography, various tyes of staining methods, enzymatic and microbiological methods, and analytical chromatography such as high-performance liquid chromatography (see, for example, Ullman, Encyclopedia of Industrial Chemistry, vol. A2, pp. 89-90 and pp. 443-613, VCH: Weinheim (1985); Fallon, A., et al., (1987) "Applications of HPLC in Biochemistry" in: Laboratory Techniques in Biochemistry and Molecular Biology, vol. 17; Rehm et al. (1993) Biotechnology, vol. 3, chapter III: "Product recovery and purification", pp. 469-714, VCH: Weinheim; Belter, P.A., et al. (1988) Bioseparations: downstream processing for Biotechnology, John Wiley and Sons; Kennedy, J.F. and Cabral, J.M.S. (1992) Recovery processes for biological Materials, John Wiley and Sons; Shaeiwitz, J.A., and Henry, J.D. (1988) Biochemical Separations, in: Ullmann's Encyclopedia of Industrial Chemistry, vol. B3; chapter 11, pp. 1-27, VCH: Weinheim; and Dechow, F.J. (1989) Separation and purification techniques in biotechnology, Noyes Publications).

In addition to the abovementioned methods, plant lipids are extracted from plant material as described by Cahoon et al. (1999) Proc. Natl. Acad. Sci. USA 96 (22):12935-12940, and Browse et al. (1986) Analytic Biochemistry 152::141-145. The qualitative

and quantitative lipid and fatty acid analysis is described by Christie, William W., Advances in Lipid Methodology, Ayr/Scotland: Oily Press (Oily Press Lipid Library; 2); Christie, William W., Gas Chromatography and Lipids. A Practical Guide - Ayr, Scotland: Oily Press, 1989, Repr. 1992, IX, 307 S. (Oily Press Lipid Library; 1); "Progress in Lipid Research, Oxford: Pergamon Press, 1 (1952) - 16 (1977) u.d.T.: Progress in the Chemistry of Fats and Other Lipids CODEN.

To determine the overall efficiency with which the compound is produced, it is also possible, in addition to measuring the fermentation end product, to analyze other components of the metabolic pathways which are used for producing the desired compounds, such as intermediates and secondary products. The analytical methods comprise measurements of the nutrient quantities in the medium (for example sugars, hydrocarbons, nitrogen sources, phosphate and other ions), measurements of the biomass composition and the growth, analysis of the production of usual metabolites via biosynthetic pathways, and measurements of gases which are generated during the fermentation process. Standard methods for these measurements are described in Applied Microbial Physiology; A Practical Approach, P.M. Rhodes and P.F. Stanbury, ed., IRL Press, pp. 103-129; 131-163 and 165-192 (ISBN: 0199635773) and references cited therein.

One example is the analysis of fatty acids (abbreviations: FAMEs, fatty acid methyl esters; GC-MS, gas liquid chromatography/mass spectrometry; TAG, triacylglycerol; TLC, thin-layer chromatography).

Unequivocal proof for the presence of fatty acid products can be obtained by the analysis of recombinant organisms following standard analytical procedures: GC, GC-MS or TLC as variously described by Christie and references therein (1997, in: Advances on Lipid Methodology, Fourth ed.: Christie, Oily Press, Dundee, 119-169; 1998, gas-chromatography/mass-spectrometry methods, Lipids 33:343-353).

Material to be analyzed can be disintegrated via sonification, glass milling, liquid nitrogen and grinding or via other applicable methods. The material has to be centrifuged after disintegration. The sediment is resuspended in Aqua dest, heated for 10 min at 100°C, cooled on ice and centrifuged again, followed by extraction in 0.5 M sulfuric acid in methanol containing 2% dimethoxypropane for 1 h at 90°C, leading to hydrolyzed oil and liquid compounds, resulting in transmethylated lipids. These fatty acid methyl esters are extracted in petrolether and finally

subjected to GC analysis using a capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 µm, 0.32 mm) at a temperature gradient beteen 170°C and 240°C for 20 min and 5 min at 240°C. The identity of resulting fatty acid methylesters has to be defined by the use of standards available from commercial sources (i.e. Sigma).

In the case of fatty acids where standards are not available molecule identity has to be shown via derivatization and subsequent GC MS analysis. For example the localization of triple bond fatty acids has to be shown via GC-MS after derivatization via 4,4-dimethoxyoxazoline derivatives (Christie, 1998, see above).

Expression constructs in heterologous microbial systems

Strains, Growth Conditions and Plasmids

Escherichia coli strain XL1 Blue MRF' kan (Stratagene) was used for sub-cloning the new elongase pPDesaturase1 from Physcomitrella patens. For functional expression of this gene we used the Saccharomyces cerevisiae strain INVSc 1 (Invitrogen Co.). E. coli was grown in Luria-Bertini broth (LB, Duchefa, Haarlem, The Netherlands) at 37°C. When necessary, ampicillin (100 mg/liter) was added and 1.5% (w/v) agar was included for solid LB media. S. cerevisiae was grown at 30°C either in YPG-medium or in complete minimal dropout uracil medium (CMdum; see in: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Albright, L.B., Coen, D.M., and Varki, A. (1995) Current Protocols in Molecular Biology, John Wiley & Sons, New York) containing either 2% (w/v) raffinose or glucose. For solid media 2% (w/v) Bacto™ agar (Difco) was included. Plasmids used for cloning and expression were pUC18 (Pharmacia) and pYES2 (Invitrogen Co.).

Example 8: Cloning and expression of PUFA-specific desaturases and elongases

For expression in plants, cDNA clones from SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31 were modified in such a way that only the coding region was amplified by means of polymerase chain reaction using two oligonucleotides. Care was taken that a consensus sequence before the start codon was maintained for efficient translation. To this end, either the base sequence ATA or AAA was chosen and introduced into the sequence before the ATG (Kozak, M. (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates

translation by eukaryotic ribosomes, Cell 44, 283-292). In addition, a restriction cleavage site was introduced before this consensus triplet, which restriction cleavage site must be compatible with the cleavage site of the target vector into which the fragment is to be cloned and with the aid of which the expression of genes in microorganisms or plants is to take place.

The PCR reaction was performed with plasmid DNA as template in a Thermocycler (Biometra) using the Pfu-DNA (Stratagene) polymerase and the following temperature programme: 3 minutes at 96°C, followed by 30 cycles with 30 seconds at 96°C, 30 seconds at 55°C and 2 minutes at 72°C, 1 cycle with 10 minutes at 72°C and stop at 4°C. The annealing temperature was varied, depending on the oligonucleotides chosen. A synthesis time of approximately one minute can be assumed per kilobase pairs DNA. Further parameters which have an effect on the PCR such as, for example, Mg ions, salt, DNA polymerase and the like are known to the specialist worker and can be varied as required.

The correct size of the amplified DNA fragment was verified by agarose-TBE gel electrophoresis. The amplified DNA was extracted from the gel using the QIAquick Gel Extraction Kit (QIAGEN) and ligated into the SmaI restriction site of the dephosphorylated vector pUC18 using the Sure Clone Ligation Kit (Pharmacia), giving rise to the pUC derivatives. After the transformation of E. coli XL1 Blue MRF' kan, a DNA miniprep (Riggs, M.G., & McLachlan, A. (1986) A simplified screening procedure for large numbers of plasmid mini-preparation. BioTechniques 4, 310-313) was carried out on ampicillin-resistant transformants, and positive clones were identified by means of BamHI restriction analysis. The sequence of the cloned PCR product was verified by resequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Weiterstadt).

Fatty acid analysis

The total fatty acids were extracted from plant seeds and analyzed by gas chromatography.
The seeds were taken up in 1% sodium methoxide in methanol and incubated for 20 minutes at RT. Thereafter, the mixture is washed with NaCl solution, and the FAMEs are taken up in 0.3 ml heptane. The samples were separated on a ZEBRON-ZB-Wax capillary column (30 m, 0.32 mm, 0.25 µm; Phenomenex) in a Hewlett Packard-6850 gas chromatograph with flame ionization detector. The oven temperature was programmed from 70°C (1 minute hold) to 200°C at a rate of 20°C/minute, then to 250°C (5 min hold) at a rate of 5°C/min and finally to 260°C at a rate of 5°C/min. Nitrogen was

used as carrier gas (4.5 ml/min at 70°C). The fatty acids were identified by comparing the retention times with those of FAME standards (SIGMA).

Expression analysis

Result of the expression of a Phaeodactylum tricornutum Δ6-acyl-lipid desaturase, a Phaeodactylum tricornutum Δ5-acyl-lipid desaturase and the delta-6-specific elongase in tobacco seeds:

Figure 2: Fatty acid profile of transgenic tobacco seeds. The plants were transformed with a triple expression cassette which expresses, under the control of the USP promoter, the delta-6-, the delta-5- and the Physcomitrella patens PpPSE1 (pARA2). 100 transgenic tobacco and linseed plants are generated, of which approximately 20% synthesize arachidonic acid in the seed.

Figure 3: Tobacco wild-type control.

Example 9: Purification of the desired product from transformed organisms

The desired product can be obtained from plant material or fungi, algae, ciliates, animal cells or from the supernatant of the above-described cultures by various methods known in the art. If the desired product is not excreted from the cells, the cells can be harvested from the culture by slow centrifugation, and the cells can be lyzed by standard techniques such as mechanical force or sonication. Plant organs can be separated mechanically from other tissue or other organs. After homogenization, the cell debris is removed by centrifugation, and the supernatant fraction, which comprises the soluble proteins, is stored for the further purification of the desired compound. If the product is excreted from desired cells, the cells are removed from the culture by slow centrifugation, and the supernatant fraction is stored for further purification.

The supernatant fraction of each purification method is subjected to chromatography with a suitable resin, the desired molecule either being retained on the chromatography resin, while many contaminations in the sample are not, or else the contaminations are retained on the resin, while the sample is not. If necessary, these chromatography steps can be repeated, using identical or different chromatography resins. The skilled worker is familiar with the selection of suitable chromatography resins and their most effective application for a particular molecule to be

purified. The purified product can be concentrated by filtration or ultrafiltration and stored at a temperature which provides maximum stability of the product.

A broad spectrum of purification methods is known in the art, and the above purification method is not intended to be limiting. These purification methods are described, for example, in Bailey, J.E., & Ollis, D.F., Biochemical Engineering Fundamentals, McGraw-Hill: New York (1986).

The identity and purity of the compounds which have been isolated can be determined by standard techniques of the art. These include high-performance liquid chromatography (HPLC), spectroscopic methods, staining methods, thin-layer chromatography, in particular thin-layer chromatography and flame ionization detection (IATROSCAN, Iatron, Tokio, Japan), NIRS, enzyme assay or microbiological methods. For an overview of these analytical methods, see: Patek et al. (1994) Appl. Environ. Microbiol. 60:133-140; Malakhova et al. (1996) Biotekhnologiya 11:27-32; and Schmidt et al. (1998) Bioprocess Engineer. 19:67-70. Ulmann's Encyclopedia of Industrial Chemistry (1996) vol. A27, VCH: Weinheim, pp. 89-90, pp. 521-540, pp. 540-547, pp. 559-566, 575-581 and pp. 581-587; Michal, G (1999) Biochemical Pathways: An Atlas of Biochemistry and Molecular Biology, John Wiley and Sons; Fallon, A., et al. (1987) Applications of HPLC in Biochemistry in: Laboratory Techniques in Biochemistry and Molecular Biology, vol. 17.

Equivalents

The skilled worker will or can recognize many equivalents of the specific embodiments according to the invention described herein by simply using routine experiments. These equivalents are intended to fall within the patent claims.